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**(57) Abstract**

The present invention relates to chimeric genes having (i) a DNA sequence encoding a product of interest, and (ii) a *drul* promoter, where said DNA sequence is heterologous to said promoter and said DNA sequence is operably linked to said promoter to enable expression of said product. The invention describes vectors, cells, plants, and fruits carrying the chimeric gene, as well as methods related thereto.

**PLANT TISSUE/STAGE SPECIFIC PROMOTERS  
FOR REGULATED EXPRESSION OF TRANSGENES IN PLANTS**

**FIELD OF THE INVENTION**

- 5       The present invention relates to the identification and characterization of tissue and/or stage specific plant promoters and compositions and methods employing such promoters.

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#### BACKGROUND OF THE INVENTION

5 In recent years recombinant DNA technology has been used to circumvent many limitations of traditional plant breeding programs. This technology has allowed workers to (i) identify and clone desirable genes (such as, genes expressing products that confer disease and insect resistance (Herreraestrella, *et al.*, 1995), (ii) transfer such genes into plants (Walkerpeach, *et al.*, 1994), and (iii) alter selected plant phenotypes by the expression of such genes (Ferro, *et al.*, 1995; Benfey, 10 *et al.*, 1990; Klee, *et al.*, 1991).

A large number of examples of plant promoters useful for the expression of selected genes in plants are now available (Zhu, *et al.*, 1995; Ni, *et al.*, 1995). These promoters have been used to drive the expression of foreign (or heterologous) genes in plants. In most cases, the 5' non-coding regions of the genes (*i.e.*, regions immediately 5' to the coding region) have been used to 15 generate chimeric genes. These regions are often referred to as promoter or transcriptional regulatory sequences. Promoters useful for the expression of a selected nucleic acid sequence in plants can be derived from plant DNA or from other sources, for example, plant viruses. In most cases, it has been demonstrated that sequences up to about 500-1500 bases allow regulated expression of genes under their control.

20 Expression of heterologous genes or selected sequences of genes in transgenic plants has typically involved the use of constitutive promoters. Exemplary plant promoters include the following: 35S Cauliflower Mosaic Virus (CaMV 35S), mannopine synthase, and octopine synthase (ocs). Such promoters have been used successfully to direct the expression of heterologous nucleic acid sequences in transformed plant tissue. However, when used to express 25 DNA sequences in transgenic plants these promoters typically provide low level, constitutive expression (*i.e.*, expression in all plant tissue).

Other promoters have been identified that allow tissue specific expression, for example, fruit specific expression, such as the E4 and E8 promoters from tomatoes (Cordes, *et al.*, 1989; Bestwick, *et al.*, 1995). Also, it has been demonstrated that nucleic acid sequences placed under 30 the regulatory control of the 5' non-coding region of the tomato 2AII gene (Van Haaren) are preferentially transcribed in developing fruit tissue. Fruit specific regulation of the kiwifruit actinidin promoter has been reported to be conserved in transgenic petunia plants (Lin, *et al.*, 1993).

**SUMMARY OF THE INVENTION**

The present invention includes a promoter that allows high-level, tissue specific expression of nucleic acid sequences placed under its regulation. Chimeric genes of the present invention have a DNA sequence encoding a product of interest under the transcriptional control of a *drul* promoter. The DNA sequence is typically heterologous to the promoter and is operably linked to the promoter to enable expression of the product. Exemplary products include, but are not limited to S-adenosylmethionine hydrolase, aminocyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, ACC synthase cosuppression molecule, thaumatin, sucrose phosphate synthase and lycopene cyclase.

In one embodiment, the promoters of the present invention can be used to reduce ethylene production in fruit cells.

In another embodiment, the DNA sequence can correspond to a pathogenesis related gene, such as polygalacturonase inhibiting protein (PGIP), glucanase and chitinase.

The promoter of the present invention can be obtained from a gene homologous to a raspberry *drul* gene or from the *drul* raspberry gene itself. An exemplary *drul* promoter sequence is SEQ ID NO:22. Smaller fragments of such a promoter region may be derived from this sequence, where the smaller fragments are effective to regulate expression of a DNA sequence under their control.

The present invention also includes the use of any of the above chimeric genes to generate a plant transformation vector. Such vectors can be used in any plant cell transformation method, including, Agrobacterium-based methods, electroporation, microinjection, and microprojectile bombardment. These vectors may form part of a plant transformation kit. Other components of the kit may include, but are not limited to, reagents useful for plant cell transformation.

In another embodiment, the present invention includes a plant cell, plant tissue, transgenic plant, fruit cell, whole fruit, seeds or calli containing any of the above-described chimeric genes.

In another aspect of the present invention, the promoters described herein are employed in a method for modifying ripening fruit of a fruit bearing plant. In this method, transgenic plants containing the chimeric gene of the present invention are grown to produce a transgenic plant bearing fruit. In this embodiment, the chimeric gene encodes a product capable of reducing ethylene biosynthesis when expressed in plant cells (e.g., S-adenosylmethionine hydrolase, aminocyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, ACC synthase cosuppression molecule). Fruit produced by these transgenic plants have a modified ripening phenotype. A

modified ripening phenotype typically refers to an alteration of the rate of ripening of a transgenic fruit relative to corresponding (*i.e.*, non-transgenic) wild-type fruit.

Further, the invention includes a method for producing a transgenic fruit-bearing plant. In this method the chimeric gene of the present invention, typically carried in an expression vector  
5 allowing selection in plant cells, is introduced into progenitor cells of selected plant. These progenitor cells are then grown to produce a transgenic plant bearing fruit. The method may further comprise isolation of a *dru1* promoter by the following steps:

- (i) selecting a probe DNA molecule containing a sequence homologous to a region of raspberry *dru1* gene DNA,
- 10 (ii) contacting the probe with a plurality of target DNA molecules derived from the genome of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe molecule and a target molecule homologous to the probe molecule,
- (iii) identifying a target molecule having a DNA sequence homologous to the raspberry *dru1* gene, and
- 15 (iv) isolating promoter sequences associated with the target molecule.

In addition, the present invention includes isolation of a *dru1* promoter by the steps just described.

The chimeric genes, vectors, products and methods of the present invention can also be produced using *dru2* promoter sequences identified essentially as described herein for *dru1*.

20 These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents representative results of polyacrylamide gel electrophoretic analysis of  
25 raspberry drupelet proteins.

Figures 2A and 2B schematically represent the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR; Kawasaki, *et al.*, 1989; Wang, *et al.*, 1990) cloning of the raspberry *dru1* gene.

Figures 3A and 3B present the genomic DNA sequence of the *dru1* gene. Indicated in the figures are a CAAT box, TATA box, ATG start codon, two exons, an intron, splicing sites, a stop  
30 codon and poly-adenylation sites.

Figure 4 presents a schematic representation of the gene organization and protein structure of *dru1*.

Figure 5 presents a Kyte-Doolittle hydrophilicity plot of the coding sequence of *dru1*. In the figure, the hydrophilicity window size = 7.

Figure 6 shows the results of RNA dot blot analysis of *dru1* RNA expression in raspberry leaf and receptacle. RNA was isolated from green, mature green, breaker & orange/ripe raspberries (corresponding to stages I, II, III, IV, respectively).

Figure 7 shows the results of a RNA hybridization study evaluating the expression of *dru1* RNA in raspberry leaf and fruit.

Figure 8 shows the results of polyacrylamide gel electrophoretic analysis of raspberry drupelet proteins obtained from drupelets at various stages of ripening.

Figures 9A and 9B present a schematic description of the details of the vector construction for pAG-4032, and

Figure 10 presents a schematic description of the details of the vector construction for pAG-4033.

#### DETAILED DESCRIPTION OF THE INVENTION

##### I. DEFINITIONS

A "chimeric gene," in the context of the present invention, typically comprises a promoter sequence operably linked to "heterologous" DNA sequences, *i.e.*, DNA sequences that encode a gene product not normally contiguous or associated with the promoter (*e.g.*, a *dru1* promoter adjacent DNA sequences encoding S-adenosylmethionine cleaving enzyme).

"*dru1* homologous genes" are defined as genes that have at least about 55% or preferably 80% global sequence homology, that is, sequence identity over a length of the polynucleotide sequence to the raspberry *dru1* polynucleotide sequences disclosed herein (*e.g.*, SEQ ID NO:10).

"Sequence homology" is determined essentially as follows. Two polynucleotide sequences of the same length (preferably, corresponding to the coding sequences of the gene) are considered to be homologous to one another, if, when they are aligned using the ALIGN program, over 55% or preferably 80% of the nucleic acids in the highest scoring alignment are identically aligned using a ktup of 1, the default parameters and the default PAM matrix (Dayhoff, 1972).

The ALIGN program is found in the FASTA version 1.7 suite of sequence comparison programs (Pearson and Lipman, 1988; Pearson, 1990; program available from William R. Pearson, Department of Biological Chemistry, Box 440, Jordan Hall, Charlottesville, VA).

Two nucleic acid fragments are considered to be "selectively hybridizable" to a polynucleotide derived from a *dru1* gene, if they are capable of specifically hybridizing to the coding sequences of the raspberry *dru1* gene or a variant thereof or of specifically priming a polymerase chain amplification reaction: (i) under typical hybridization and wash conditions, as described, for example, in Maniatis, *et al.* (1982), pages 320-328, and 382-389. Examples of such hybridization conditions are also given in Examples 8 and 9; (ii) using reduced stringency wash

conditions that allow at most about 25-30% basepair mismatches, for example: 2 × SSC, 0.1% SDS, room temperature twice, 30 minutes each; then 2 × SSC, 0.1% SDS, 37°C. once, 30 minutes; then 2 × SSC room temperature twice, 10 minutes each, or (iii) selecting primers for use in typical polymerase chain reactions (PCR) under standard conditions (for example, in Saiki, 5 *et al.*, 1988), which result in specific amplification of sequences of *drul* or its variants.

Preferably, highly homologous nucleic acid strands contain less than 20-40% basepair mismatches, even more preferably less than 5-20% basepair mismatches. These degrees of homology can be selected by using wash conditions of appropriate stringency for identification of clones from gene libraries (or other sources of genetic material), as is well known in the art.

10 A "*drul* encoded polypeptide" is defined herein as any polypeptide homologous to a *drul* encoded polypeptide. In one embodiment, a polypeptide is homologous to a *drul* encoded polypeptide if it is encoded by nucleic acid that selectively hybridizes to sequences of *drul* or its variants.

In another embodiment, a polypeptide is homologous to a *drul* encoded polypeptide if it is 15 encoded by *drul* or its variants, as defined above, polypeptides of this group are typically larger than 15, preferable 25, or more preferable 35, contiguous amino acids. Further, for polypeptides longer than about 60 amino acids, sequence comparisons for the purpose of determining "polypeptide homology" are performed using the local alignment program LALIGN. The polypeptide sequence is compared against the *drul* amino acid sequence or any of its variants, as 20 defined above, using the LALIGN program with a ktup of 1, default parameters and the default PAM.

Any polypeptide with an optimal alignment longer than 60 amino acids and greater than 55% or preferably 80% of identically aligned amino acids is considered to be a "homologous polypeptide." The LALIGN program is found in the FASTA version 1.7 suite of sequence 25 comparison programs (Pearson and Lipman, 1988; Pearson, 1990; program available from William R. Pearson, Department of Biological Chemistry, Box 440, Jordan Hall, Charlottesville, VA).

A polynucleotide is "derived from" *drul* if it has the same or substantially the same basepair sequence as a region of the *drul* protein coding sequence, cDNA of *drul* or complements thereof, or if it displays homology as noted above.

30 A polypeptide or polypeptide "fragment" is "derived from" *drul* if it is (i) encoded by a *drul* gene, or (ii) displays homology to *drul* encoded polypeptides as noted above.

In the context of the present invention, the phrase "nucleic acid sequences," when referring to sequences which encode a protein, polypeptide, or peptide, is meant to include degenerative



nucleic acid sequences which encode homologous protein, polypeptide or peptide sequences as well as the disclosed sequence.

A "modified ripening" phenotype typically refers to an alteration of the rate of ripening of a transgenic fruit relative to corresponding wild-type fruit, such as, for example, delayed ripening fruit (*i.e.*, ripening takes longer than corresponding wild-type fruit) or suspension of the fruit's ability to complete the ripening process.

A "product" encoded by a DNA molecule includes, for example, an RNA molecule or a polypeptide.

## II. *DRU1* PROTEIN IDENTIFICATION, PURIFICATION AND SEQUENCE DETERMINATION.

The present invention relates to the cloning of a gene expressed at very high levels in ripening fruit, exemplified by the *dru1* gene from raspberries. Expression directed by the *dru1* promoter described herein is fruit specific and active during fruit ripening.

Protein(s) such as those produced by raspberry are typically analyzed by gel electrophoresis. A coomassie blue-stained SDS polyacrylamide gel of soluble drupelet proteins is shown in Figure 1 (Example 1). Two highly abundant proteins isolable from raspberries are observed at approximately 17 and 15 kd, and are referred to herein as drupe1 and drupe2, respectively. The amount of drupe1 and drupe2 relative to the total amount of soluble protein can be determined, for example, by scanning densitometry. Scanning densitometry analysis of the gel illustrated in Fig. 1 indicates that drupe1 and drupe2 comprise approximately 23 and 37%, respectively, of the total soluble protein in raspberry drupelets. As a result of this determination (*i.e.*, the high levels of drupe1 and drupe2), purification and sequencing of drupe1 and drupe2 can be carried out, for example, by using a direct western blot approach.

In carrying out a western blot analysis, total drupelet proteins are western blotted to PDVF membrane (Example 1) and the regions corresponding to drupe1 and drupe2 are subjected to N-terminal amino acid sequence analysis. The drupe1 sample yields a thirty amino acid N-terminal sequence (Example 1). The amino terminal drupe1 sequence is presented herein as SEQ ID NO:1.

## III. CLONING *DRU1* ENCODING SEQUENCES.

### A. RT-PCR AND CLONING OF A *DRU1* CDNA CLONE.

The entire procedure for cloning *dru1*, from cDNA synthesis to inverse PCR of a genomic copy of the gene, is shown schematically in Figures 2A and 2B.

In carrying out the cloning procedure, mature green raspberry drupelet mRNA is prepared as described in Example 2 and used as template in a cDNA synthesis reaction. The reaction is primed using the dRANDOM primer shown in Figures 2A and 2B. The resulting cDNA

(Example 2) is subjected to a standard PCR reaction using primers corresponding to a portion of the dRANDOM primer and a 512-fold degenerate primer (Drupe 20) based on the drupe1 amino terminal sequence (Example 3).

The PCR amplification products are then analyzed. Products from the above PCR reaction include a 710 bp product that is agarose gel purified and subcloned into pCRII (Example 3). Subsequent sequence analysis of several of these clones allows identification of those clones whose sequence encodes a protein matching the amino terminal sequence of drupe1.

#### B. INVERSE PCR CLONING OF A GENOMIC COPY OF THE *DRU1* GENE

In this approach to cloning the *drul* gene, genomic raspberry DNA is used in a PCR reaction using primers internal to the cDNA sequence obtained as described above (Example 4). This reaction produces a genomic clone of the *drul* gene containing most of the protein coding region. A single intron was identified from the subsequent sequence analysis of this clone (Figure 3B). An inverse PCR strategy may be employed to characterize and sequence the 5' region of the gene containing the *drul* promoter (Example 5). Figures 2A and 2B show schematically how this may be accomplished.

In characterizing the 5' flanking region of *drul* genomic DNA utilizing inverse PCR techniques, raspberry genomic DNA is digested with *NsiI* and ligated under dilute conditions to allow circularization of the restriction fragments. The ligated DNA is then subjected to PCR amplification using primers internal to the *drul* coding sequence and oriented in opposite directions from each other. This produces a PCR reaction product containing part of the first exon and 1.35 kb of the promoter. Subsequent sequence analysis of this clone in combination with sequence information from the previously described clones produces the complete *drul* sequence (SEQ ID NO:12).

#### C. SEQUENCE DETERMINATION AND EVALUATION OF GENE EXPRESSION PATTERNS.

The *drul* gene (SEQ ID NO:12) encodes a protein with the predicted amino acid sequence presented as SEQ ID NO:13. The predicted molecular weight for this protein is 17,088, which agrees closely with the 17kd molecular weight determined by gel electrophoresis (see Figure 1) of total drupelet protein. The *drul* protein is relatively acidic with a predicted pI of 4.8. Nucleic acid and protein homology searches of the current sequence databases can be carried out to look for significant matches. For *drul*, nucleic acid and protein homology searches of the current sequence databases produced no significant matches. This result supports the original observation made with the amino terminal sequence of the protein that drupe1 is a novel protein.

The gene expression pattern of *drul* can be also be evaluated at the RNA and protein levels to confirm the tissue specificity of the promoter. Northern dot blots, Figures 6 and 7, of total

RNA from raspberry leaf and receptacles at different ripening stages indicate a tissue and stage specific gene expression pattern. This can be confirmed by comparison of northern blots of total RNA from various other plant tissues. The tissue and stage specific gene expression pattern of *dru1* was confirmed on northern blots of total RNA from leaf, receptacles, and drupelets (see  
5 Figures 6 and 7). In both cases, no *dru1* expression is observed in leaf RNA. The RNA expression pattern in receptacles is temporally regulated while in drupelets it is fully expressed at the two stages (*i.e.*, green and ripe) analyzed.

A protein gel of drupelet lysates from different ripening stages can also be carried out to further support stage specific expression of *dru1*. As illustrated in Figure 8, electrophoretic  
10 analysis of raspberry drupelet proteins obtained from drupelets at various stages of ripening (*i.e.*, green, mature green, breaker, orange, and ripe) further supports a stage specific expression pattern in drupelets (Figure 8).

The level of both protein and mRNA expression of *dru1* is very high. Although not wishing to be bound by any particular mechanism for the observations described herein, there are several  
15 possible mechanisms that may contribute to such high level protein and mRNA expression. One mechanistic possibility is that the *dru1* promoter is a strong promoter. Data supporting this mechanism for protein and mRNA expression is discussed above.

#### D. PROMOTER ISOLATION AND CONSTRUCTION OF CHIMERIC GENES.

Characterization of the *dru1* genomic clone allows isolation of the *dru1* promoter. The  
20 promoter can then be used to regulate expression of heterologous genes. An exemplary *dru1* promoter has the sequence presented as SEQ ID NO:22.

In support of the present invention, two exemplary chimeric genes containing a *dru1* promoter sequence operably linked to a heterologous DNA sequence, were constructed, *dru1*pro:-SAMase and *dru1*pro:PGIP (Example 7). S-adenosylmethionine hydrolase (SAMase) and poly-  
25 galacturonase inhibiting protein (PGIP) confer ethylene control and fungal resistance, respectively, in transgenic plants. Both proteins have been predicted to function more efficiently if expressed (i) in high levels and (ii) in a tissue specific manner. Accordingly, the *dru1* promoter represents an ideal promoter to satisfy this objective.

Construction of *Agrobacterium* binary vectors, pAG-4032 and pAG-4033, containing the two  
30 representative chimeric genes described above, can be performed as described in Example 7 (schematically represented in Figures 9 and 10, *dru1*pro:SAMase and *dru1*pro:PGIP, respectively).

#### IV. IDENTIFICATION OF PLANT *DRU1* PROMOTERS

The present invention provides for the use of *dru1* promoters from species other than raspberry. Such promoters are useful for the generation of vector constructs containing heterolo-

gous genes. Southern blot experiments are used to demonstrate the presence of DNA molecules having significant sequence identity (*i.e.*, typically greater than 55%, more preferably greater than 80% identity using standard sequence comparison programs) with the raspberry *drul* gene in, for example, strawberry, peach or plum. Similar Southern blot analyses may be performed on other  
5 fruit-bearing plants to identify additional *drul* genes.

A Southern blot analysis used herein is detailed in Example 8. *drul* homologues are identified in a Southern blot of the genomic DNA of the plants listed above probed with a labelled DNA fragment containing the coding sequence of the raspberry *drul* gene.

The probe is selected to contain the coding sequence of *drul*, rather than the promoter  
10 sequence, because coding sequences are typically more conserved from species to species than are promoter sequences. In the experiments detailed in Examples 8 and 9, probe molecules are generated from raspberry genomic DNA using primer-specific amplification (Mullis, 1987; Mullis, *et al.*, 1987). The oligonucleotide primers are selected such that the amplified region includes the entire coding sequence of the raspberry *drul* gene. Primers may also be selected to amplify only  
15 a selected region of the raspberry *drul* gene.

Alternatively, a probe can be made by isolating restriction-digest fragments containing the sequence of interest from plasmid DNA.

The probe is labeled with a detectable moiety to enable subsequent identification of homologous target molecules. Exemplary labeling moieties include radioactive nucleotides, such  
20 as <sup>32</sup>P-labeled nucleotides, digoxigenin-labeled nucleotides, biotinylated nucleotides, and the like, available from commercial sources.

In the case of primer-amplified probes, labeled nucleotides may be directly incorporated into the probe during the amplification process. Probe molecules derived from DNA that has already been isolated, such as restriction-digest fragments from plasmid DNA, are typically end-labeled  
25 (Ausubel, *et al.*, 1992).

Target molecules, such as *HindIII* DNA fragments from the genomes of the above-listed plants, are electrophoresed on a gel, blotted, and immobilized onto a nylon or nitrocellulose filter. Labeled probe molecules are then contacted with the target molecules under conditions favoring specific hybridization between the probe molecules and target molecules homologous to the probe  
30 molecules (Maniatis, *et al.*, 1982; Sambrook, *et al.*, 1989; Ausubel, *et al.*, 1992).

Conditions favoring specific hybridization are referred to as moderately to highly stringent, and are affected primarily by the salt concentration and temperature of the wash buffer (Ausubel, *et al.*, 1992; Sambrook, *et al.*, 1989). Conditions such as those used in the final wash in Example 9 are typically classified as moderately stringent, due to the low salt concentration, and are

expected to preserve only specific hybridization interactions, allowing the identification and isolation of homologous genes in different plant species.

Following contacting, hybridization, and washing, target molecules with sequences homologous to the probe are identified by detecting the label on the probe. The label may be detected directly, for example, as in a radioactive label detected on autoradiograms, or it may be detected with a secondary moiety, for example, fluorescently-labeled streptavidin binding to a biotinylated probe.

Following the identification of plants containing *drul* genes, the DNA containing the desired genes, including the promoter regions, may be isolated from the respective species, by, for example, the methods described herein for the isolation of the raspberry *drul* gene.

Typically, a library of interest (*e.g.*, genomic or cDNA) is screened with a probe containing sequences corresponding to the coding sequence of a known *drul* gene, such as the raspberry *drul* gene (Example 9). The screening is done using known methods (Ausubel, *et al.*, 1992; Sambrook, *et al.*, 1989), essentially as described above.

Positive plaques or colonies are isolated, and the insert DNA is sequenced and compared to known *drul* sequences. Clones containing inserts with sequences corresponding to genes homologous to raspberry *drul* are identified and, if necessary, used to obtain additional clones until the promoter region of interest is isolated.

Variants of the *drul* promoter may be isolated from different raspberry cultivars and from other plants by the methods described above. A reporter gene, such as GUS ( $\beta$ -glucuronidase), can be used to test tissue and/or stage specific (*e.g.*, stages of fruit ripening) regulatable expression from such promoters. Expression of GUS protein can be easily measured by fluorometric, spectrophotometric or histochemical assays (Jefferson, 1987a, 1987b).

Further, using chimeric genes containing *drul* promoter sequences operably linked to reporter gene sequences, DNA sequences corresponding to regulatory domains can be identified using, for example, deletion analysis (Benfey, *et al.*, 1990). For example, the *drul* promoter sequence presented as SEQ ID NO:22 can be functionally linked to the GUS reporter gene. Deletion analysis can then be carried out by standard methods (Ausubel, *et al.*, 1992; Maniatis, *et al.*, 1982; Sambrook, *et al.*). Alternatively, regions of the *drul* promoter sequence can be amplified using sequence-specific primers in PCR. These amplified fragments can then be inserted 5' to the GUS coding sequences and the resulting expression patterns evaluated.

## V. PLANT TRANSFORMATION AND THE GENERATION OF TRANSGENIC PLANTS.

### A. THE VECTORS OF THE PRESENT INVENTION.

Plant transformation vectors, containing *drul* promoter/transcription-regulatory sequences, are constructed according to methods known in the art (see, for example, Houck and Pear, 1990, and Becker, *et al.*, 1992).

The present invention provides vectors suitable for the transformation of plants. The vectors, chimeric genes and DNA constructs of the present invention are also useful for the expression of heterologous genes. Transgenic plants, and their fruit products, carrying the chimeric genes of the present invention, may be a useful source of recombinantly-expressed material.

10 In one embodiment, the chimeric genes of the present invention have two components: (i) a promoter derived from a *drul* gene, and (ii) a heterologous DNA sequence encoding a desirable product.

The vectors of the present invention may be constructed to carry an expression cassette containing an insertion site for DNA coding sequences of interest. The transcription of such inserted DNA is then under the control of a suitable *drul* promoter (*i.e.*, raspberry *drul* gene promoter or homologs thereof).

Such expression cassettes may have single or multiple transcription termination signals at the coding-3'-end of the DNA sequence being expressed. Such 3' sequences may include transcription termination sequences derived from the 3' non-coding region of the *drul* gene encoded mRNA.

20 The expression cassette may also include, for example, DNA sequences encoding (i) a leader sequence (*e.g.*, to allow secretion or vacuolar targeting), and (ii) translation termination signals.

Further, the vectors of the present invention may include selectable markers for use in plant cells (such as, the *nptII* kanamycin resistance gene). The vectors may also include sequences that allow their selection and propagation in a secondary host, such as, sequences containing an origin of replication and a selectable marker. Typical secondary hosts include bacteria and yeast. In one embodiment, the secondary host is *Escherichia coli*, the origin of replication is a *colE1*-type, and the selectable marker is a gene encoding ampicillin resistance. Such sequences are well known in the art and are commercially available as well (*e.g.*, Clontech, Palo Alto, CA; Stratagene, La Jolla, CA).

30 The vectors of the present invention may also be modified to intermediate plant transformation plasmids that contain a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens*, and chimeric genes or expression cassettes (described above). Further, the vectors of the invention may comprise a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*. Other suitable vectors may be constructed using

the promoters of the present invention and standard plant transformation vectors, which are available both commercially (Clontech, Palo Alto, CA) and from academic sources (Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, NJ).

The vectors of the present invention are useful for tissue and/or stage-specific expression of nucleic acid coding sequences in plant cells. For example, a selected peptide or polypeptide coding sequence can be inserted in an expression cassette of a vector of the present invention. The vector is then transformed into host cells, the host cells cultured under conditions to allow the expression of the protein coding sequences, and the expressed peptide or polypeptide isolated from the cells. Transformed progenitor cells can also be used to produce transgenic plants bearing fruit.

In one aspect of the invention, fruit produced by such transgenic plants has a reduced level of ethylene synthesis by the fruit. The fruit then demonstrates a modified ripening phenotype.

The vectors, chimeric genes and DNA constructs of the present invention can be sold individually or in kits for use in plant cell transformation and the subsequent generation of transgenic plants.

#### B. HETEROLOGOUS GENES.

The methods and results described herein demonstrate the ability to provide tissue and/or stage specific regulation of gene expression in transgenic plants. The tissue and/or stage-specific promoters of the present invention include a region of DNA that regulates transcription of the immediately adjacent (downstream) gene to a specific plant tissue. According to methods of the present invention, heterologous genes are linked to the promoters of the present invention. Exemplary heterologous gene for the transformation of plants include genes whose products are effective to reduce ethylene biosynthesis in specific tissues of those plants, e.g. the fruits. Some of these genes, including AdoMetase, are discussed above.

Other genes of interest that could be used in conjunction with the *dru1* promoter include, but are not limited to, the following: other ripening modification genes, in addition to AdoMetase, such as, aminocyclopropane-1-carboxylic acid (ACC) deaminase (Klee, *et al.*, 1991; Sheehy, *et al.*, 1991), which degrades precursors of ethylene biosynthesis; ripening modification through the use of gene inactivation methods including antisense or cosuppression affecting genes of the ethylene biosynthetic pathway such as the genes encoding ACC synthase (Sato and Theologis, 1989) or ACC oxidase (Hamilton, *et al.*, 1990). Further, the usefulness of genes involved in conferring fungal resistance (*e.g.*, the polygalacturonase inhibiting protein (PGIP) from *Phaseolus vulgaris* (Toubart, *et al.*, 1992) and modified forms of plant glucanase, chitinase and other pathogenesis related (PR) genes (Melchers, *et al.*, 1993, 1994; Ponstein, *et al.*, 1994; Woloshuk, *et al.*, 1991) would be improved when used with a high-level, fruit-specific promoter such as *dru1*.

In addition, antisense or cosuppression genes encoding proteins responsible for degradative processes in the fruit may also be used in conjunction with the promoters of the present invention. Examples of genes of this type include polygalacturonase, cellulase, and pectin methyl esterase (Schuch, 1994). Use of the promoters of the present invention targets inhibition of the specific  
5 degradation process to only ripening fruit.

Other gene products which may be useful to express using the promoters of the present invention include genes encoding (i) flavor (*e.g.*, thaumatin; GENBANK) or color modification (*e.g.*, products that modify lycopene synthesis, for example, arabidopsis lycopene cyclase; GENBANK), (ii) enzymes or other catalytic products (such as, ribozymes or catalytic antibodies)  
10 that modify plant cell processes, (iii) gene products that affect ethylene production, such as antisense molecules, enzymes that degrade precursors of ethylene biosynthesis, catalytic products or cosuppression molecules, (iv) alternative fungal control genes, and (v) sucrose accumulating genes, such as the sucrose phosphate synthase gene (GENBANK) from corn.

Further, it is useful to restrict expression of some genes to specific tissues, such as the fruit—  
15 for example, any gene that would be deleterious to the plant if it were expressed constitutively. Such genes would include genes which encoded degradative enzymes that deplete necessary metabolites. Derivatives of the *dru1* promoter region can be used as on/off switches for the tissue and/or stage-specific expression of genes whose expression is under their control.

#### C. METHODS OF TRANSFORMING PLANTS

20 A number of methods, in addition to *Agrobacterium*-based methods, may be employed to elicit transformation of plant progenitor cells, such as electroporation, microinjection, and microprojectile bombardment. These methods are well known in the art (Comai and Coning, 1993; Klein, *et al.*, 1988; Miki, *et al.* 1987; Bellini, *et al.*, 1989) and provide the means to introduce selected DNA into plant genomes: such DNA may include a DNA cassette which con-  
25 sists of a *dru1* gene promoter functionally adjacent to heterologous sequences encoding a desired product, for example, AdoMetase coding sequences. Transformants and resulting transgenic cells and transgenic plants are identified and evaluated by standard methods (Mathews, *et al.*, 1995).

#### D. EXPRESSION IN HETEROLOGOUS PLANT SYSTEMS.

Experiments performed in support of the present invention demonstrate the versatility of the  
30 chimeric gene constructs of the invention. The vector constructs of the present invention can be used for transformation and expression of heterologous sequences in transgenic plants independent of the original plant source for the promoter sequence. Further, the expression mediated by the promoter appears to be tissue and/or stage-specific even in heterologous plants. Accordingly, the vectors, chimeric genes and DNA constructs of the present invention are useful for transformation



of species of fruit-bearing plants, where such plants are different species than the plant source of the promoter sequences.

## VI. UTILITY

The present invention relates to the cloning of a gene expressed at very high levels in ripening fruit, *e.g.*, raspberries. The gene isolated from raspberry was designated *dru1* and encodes a protein with a molecular weight of 17kd. Analysis of protein expression in raspberry drupelets indicates *dru1* comprises at least 23% of the total protein. Combined with *dru2*, an apparently similar 15kd protein expressed at even higher levels, these two proteins comprise at least 65% of the protein in raspberry drupelets. This is an unusually high level of gene expression for any plant tissue other than seed storage proteins.

Experiments performed in support of the present invention demonstrate that the gene expression patterns of the mature protein and mRNA encoded by the *dru1* gene are strictly regulated to the receptacles and drupelets of ripening raspberries. Accordingly, use of the *dru1* promoter allows the targeting of foreign gene expression to fruit tissues (*i.e.*, when such foreign gene is placed under the control of the *dru1* promoter). The *dru2* gene and corresponding promoter regions may be characterized essentially as described herein for *dru1*.

*dru1* can be cloned as described above employing N-terminal amino acid sequence information and corresponding degenerate PCR primers used in RT-PCR reactions to obtain a cDNA clone. Inverse PCR can be used to obtain a genomic clone of the gene including the *dru1* promoter.

The *dru1* gene represents an import discovery in the field of agricultural biotechnology from several standpoints. First, the *dru1* promoter can be used to express any heterologous gene whose function would be enhanced or enabled by a high level, tissue specific promoter. Two examples of such genes have been described herein: the SAMase gene (for control of ethylene synthesis and therefore ripening control), and the PGIP gene (for fungal control, specifically gray mold or *Botrytis cinerea*). Other exemplary genes are described above.

Second, the use of this promoter cannot be considered limited to raspberries. The raspberry is essentially a miniature drupe fruit so it is likely that the *dru1* promoter will function in other drupe fruits. The constructs and methods of the present invention are applicable to all higher plants including, but not limited to, the following: Berry-like fruits, for example, *Vitis* (grapes), *Fragaria* (strawberries), *Rubus* (raspberries, blackberries, loganberries), *Ribes* (currants and gooseberries), *Vaccinium*, (blueberries, bilberries, whortleberries, cranberries), *Actinida* (kiwifruit and Chinese gooseberry). Further, other drupe fruits, including, but not limited to, *Malus* (apple), *Pyrus* (pears), most members of the *Prunus* genera, sapota, mango, avocado, apricot, peaches,

cherries, plums, and nectarines. Control of ethylene production via, for example, a *druI*pro:SAMase chimera would be valuable in climacteric fruits (*e.g.*, peaches and plums) which suffer from over-ripening in post-harvest distribution systems.

Further, the results described herein that the *druI* gene is expressed in receptacles makes it likely that the promoter will function in strawberries. The strawberry fruit is a swollen receptacle that is indistinguishable, from a botanical standpoint, from the raspberry receptacle. All drupe fruits (*e.g.*, raspberries) and strawberries are members of the Rosacea genera thus making the *druI* promoter likely to function as a fruit specific promoter in heterologous species of this genera.

The present invention provides compositions and methods to regulate plant cell expression of any gene in a tissue and/or stage-specific manner. In one embodiment, the invention teaches the use of the *druI* tissue and stage-specific promoter whose expression is induced during fruit ripening.

In one embodiment, the promoters of the present invention can be used to regulate cellular production of ethylene. In this embodiment, a gene whose product results in a reduction of ethylene synthesis is operably linked to a *druI* promoter (creating a chimeric gene). When the chimeric gene is present in fruit cells, the result is fruit having a modified ripening phenotype relative to wild-type (non-transgenic) fruit.

Exemplary gene products that result in reduction of ethylene synthesis include, but are not limited to the following: S-adenosylmethionine hydrolase; 1-aminocyclopropane-1-carboxylate deaminase (Klee, *et al.*, 1991; Sheehy, *et al.*, 1991); the ACC synthase gene in an antisense or cosuppression configuration (Oeller, *et al.*, 1991; Van der Straeten, *et al.*, 1990); and the ACC oxidase gene in either an antisense or cosuppression configuration (Hamilton, *et al.*, 1990; Holdsworth, *et al.*, 1987). Cosuppression has been described by Jorgensen, *et al.* (1991, 1993).

Other gene products that may be useful in the reduction of ethylene biosynthesis include catalytic antibodies and ribozyme molecules.

The present invention provides, in one aspect, nucleic acid constructs suitable for transforming plants with heterologous genes under the control of a *druI* promoter. In one embodiment, the plant is a fruit-bearing plant, and the heterologous gene is a gene effective to reduce ethylene biosynthesis in fruit from the plant.

Experiments performed in support of the present invention describe the construction of chimeric gene constructs containing the Adometase (or SAMase) gene, isolated from bacteriophage T3 (Ferro, *et al.* (1995); Hughes, *et al.*, 1987).

The *druI* promoter may be employed in vector constructs used to produce transgenic plants, such as transgenic raspberries. For example, a vector engineered according to methods of the

present invention containing the *drul* promoter connected to the AdoMetase gene (*e.g.* vector pAG-4032), may be used to produce transgenic raspberries, strawberries, peaches, plums and the like. The AdoMetase gene will be expressed in the fruit of these transgenic plants and will delay ripening. An advantage of the method of the present invention compared to other ripening inhibition approaches, namely antisense and/or cosuppression of ACC oxidase and ACC synthase, is a savings of time and resources involved in vector construction, since the same vector can be used to transform many different plant types.

Alternatively, *drul* promoter sequences may be isolated from the same type of plant that is to be transformed, and incorporated into the vector constructs used to perform the transformations. For example, a strawberry *drul* promoter may be connected to a heterologous gene, such as the AdoMetase gene, and used to transform strawberries.

The following examples illustrate, but in no way are intended to limit the present invention.

#### MATERIALS AND METHODS

Oligonucleotides were synthesized by Operon Technologies, Inc., Alameda, CA.

Generally, the nomenclature and laboratory procedures with respect to standard recombinant DNA technology can be found in Sambrook, *et al.*, (1989); Wang, *et al.* (1989); Kawasaki, *et al.* (1989), and in Gelvin and Schilperoot (1988). Other general references are provided throughout this document. The procedures therein are known in the art and are only provided for convenient reference.

#### EXAMPLES

##### EXAMPLE 1

##### Raspberry Drupelet Protein Characterization and Purification

##### A. PROTEIN LYSATE PREPARATION AND GEL ELECTROPHORESIS.

Using a mortar and pestle containing liquid nitrogen, a raspberry protein sample was prepared by grinding the frozen drupes of one whole berry into a fine powder. Sample buffer (0.05 M Tris, pH 6.8, 1% SDS, 5% beta-mercaptoethanol, 10% glycerol; Laemmli, 1970) was added (900  $\mu$ l) to the tissue and the sample mixed by vortexing. The sample was heated for 10 minutes at 90-95°C and centrifuged at 14K rpm, 4°C for 10 minutes. The supernatant was removed from the insoluble debris pellet and stored at -20°C.

Drupelet proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) combined with coomassie blue staining using standard procedures for these steps. A coomassie blue-stained SDS polyacrylamide gel of soluble drupelet proteins is shown in Figure 1. In the figure: lane 1, molecular weight markers (BioRad, Richmond, CA), lanes 2, 3

and 5 each contain 9  $\mu$ g of raspberry drupelet protein lysate prepared separately from individual fruit. Lane 4 had a higher amount of lysate.

Two highly abundant proteins were observed at approximately 17 and 15 kd and were named drupe1 and drupe2, respectively. In Figure 1 these two proteins are indicated by arrows. Scanning densitometry analysis of this gel indicated drupe1 and drupe2 comprise approximately 23 and 37%, respectively, of the total soluble protein in raspberry drupelets. As a result of this determination, a direct western blot approach to purification and sequencing of the protein was taken.

#### B. PROTEIN BLOT FOR SEQUENCING.

10 A protein blot (Applied Biosystems, Inc. User Bulletin Number 58; Ausubel, *et al.*, 1992) was prepared using the raspberry protein lysate described above. Varying amounts of raspberry protein lysate (12-36  $\mu$ g/well) were loaded on a 10 well 18% SDS-PAGE minigel (1.5 mm thick) with 4.5% stacker and electrophoresed at 100 volts in 25 mM Tris, 192 mM glycine, 0.1% SDS buffer for 2-2.5 hours.

15 Proteins were transblotted onto Applied BioSystem's "PROBLOTT" polyvinylidene difluoride (PVDF) membrane in a 25 mM Tris, 192 mM glycine, 10% methanol buffer at 90 volts for 2 hours at 4°C. After protein transfer, the blot was Coomassie blue stained and the 15 and 17 kilodalton (kd) protein bands were located on the blot and cut out. N-terminal sequencing of the proteins was carried out at the W.M. Keck Foundation, Biotechnology Resource Laboratory in  
20 New Haven, CT.

The drupe1 sample yielded a thirty amino acid N-terminal sequence. The drupe2 sample did not yield useful sequence information likely due to a blocked amino terminus. The amino terminal drupe1 sequence is presented as SEQ ID NO:1. This 30 amino acid drupe1 sequence was compared to the protein database using BLAST searching; no significant matches were found  
25 indicating that drupe1 is a novel protein.

### EXAMPLE 2

#### Recovering a cDNA Clone Corresponding to the Drupe1 Protein

##### A. DRUPELET TOTAL RNA PREPARATION.

30 RNA was extracted from mature green raspberry drupelets. Four mature green raspberry fruit, which had been picked in season and stored at -80°C, were used to extract RNA. The estimated weight of the drupelets was 12 grams. In a cold mortar, which contained liquid nitrogen, the whole berries were fractured by tapping them with a pestle. The drupelets were separated from the receptacles. The receptacles were removed from the mortar and discarded. The drupelets were ground to a powder in the mortar, adding liquid nitrogen as necessary to keep

the tissue frozen. The seeds were purposefully left intact. Homogenization buffer, 2 ml/gram of tissue, was used to extract the RNA. [Homogenization buffer: 200 mM Tris-HCl pH 8.5, 300 mM LiCl, 10 mM Na<sub>2</sub>EDTA, 1% (w/v) sodium deoxycholate, 1.5% (w/v) sodium dodecyl sulfate (SDS), 8.5% (w/v) insoluble polyvinylpyrrolidone (PVPP), 1% (v/v) NP-40, 1 mM aurintricarboxylic acid (ATA), 5 mM thiourea, and 10 mM dithiothreitol (DTT); the last three components were added after autoclaving].

The frozen powdered drupelet tissue was added to the buffer in 3 to 5 portions, vortexing between additions until all tissue was moistened. The tissue plus buffer solution (referred to herein as the pulp) was diluted 1:1 with sterile water and 0.75 volumes of homogenization buffer were added to the diluted pulp. The sample was incubated at 65°C for 10 to 15 minutes, followed by centrifugation in a swinging bucket rotor at 9000 g for 15 minutes at 4°C. The supernatant was transferred to a clean tube. Cesium chloride (CsCl) was added to the supernatant at 0.2 g/ml. The sample was mixed until the CsCl dissolved.

A 4 ml cushion was dispensed into a Beckman 1 × 3.5 inch polyallomer ultracentrifuge tube (cushion: 5.7 M CsCl, 10 mM Tris-HCl, pH 8.0, 1 mM Na<sub>2</sub>EDTA, pH 8.). The sample was gently layered on top of the cushion. The sample was spun in a Beckman L8-80M ultracentrifuge with a SW 28 rotor at 23,000 rpm at 20°C for 20 hours. After removing the sample from the ultracentrifuge the supernatant was pulled off the sample by using a drawn Pasteur pipette attached to an aspirator. A clear lens-like pellet was visible in the bottom of the tube.

The pellet was dissolved in 500 µl SSTE and transferred to a microfuge tube (SSTE: 0.8 M NaCl, 0.4% SDS, 10 mM Tris-HCl, pH 8.0 and 1 mM Na<sub>2</sub>EDTA, pH 8). The sample was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1). To precipitate the RNA, 2.5 volumes ethanol were added to the aqueous phase. The sample was collected by centrifugation, washed two times with 75% ethanol and resuspended in 100 µl TE. The yield was 1.6 mg. The RNA was re-precipitated with 1/9 volume 3 M Sodium Acetate and 3 volumes ethanol for storage at -20°C.

#### B. DRUPELET MRNA PREPARATION.

The isolation of mRNA from mature green raspberry drupelet total RNA was performed using the "STRAIGHT A'S" mRNA isolation system (Novagen, Madison, WI) according to the manufacturer's instructions. mRNA was isolated from the 1.6 mg of total RNA extracted from mature green raspberry drupelets described above. The yield of mRNA from this procedure was 6.6 µg.

#### C. MAKING cDNA FROM GREEN RASPBERRY DRUPELET MRNA.

The mRNA from mature green raspberry drupelet RNA was used as the template for cDNA synthesis. The primer for the cDNA reactions was dRANDOM (SEQ ID NO:2; synthesized by Operon Technologies, Inc., Alameda, CA). The oligo(dT) region hybridized to the poly(A) region of the mRNA pool. The other 15 nucleotides created a 5' overhang that was used to facilitate PCR amplification at a later step in the cloning process.

The following reaction mixture was assembled for the cDNA synthesis reaction: H<sub>2</sub>O, 10.2  $\mu$ l; 250 ng mRNA, 0.8  $\mu$ l; 5  $\times$  BRL RT buffer (BRL, Bethesda, MD), 4.0  $\mu$ l; 100 mM DTT (dithiothreitol - BRL, Bethesda, MD), 0.2  $\mu$ l; "RNAGuard" (23.4 U/ $\mu$ l; an RNase inhibitor from Pharmacia, Piscataway, NJ), 0.5  $\mu$ l; dNTP's (2.5 mM each), 2.0  $\mu$ l; 50  $\mu$ M primer, 1.0  $\mu$ l; [<sup>32</sup>P]dCTP (3000 Ci/mmol; DuPont/NEN, Boston, MA), 1.0  $\mu$ l; and AMV-reverse-transcriptase (38 U/ $\mu$ l; Life Sciences, Inc., St. Petersburg, Florida), 0.3  $\mu$ l. The cDNA reaction was performed by combining mRNA and water for the reaction and heating to 65°C for 3 minutes. The mixture was cooled on ice and microfuged (to collect condensation). The remaining reaction components were then added.

After incubating at 42°C for 1 hour the cDNA reactions were moved to ice and stored at 4°C prior to their use in PCR reactions.

### EXAMPLE 3

#### PCR Amplification and Cloning of the cDNA *dru1* Fragment

A degenerate PCR primer, Drupe20, was designed for the 5' end of the cDNA based on the reverse translation of the *dru1* protein sequence. A section of the known amino acid sequence of *dru1* (SEQ ID NO:3) was chosen for its proximity to the amino terminus and for the relatively low level of degeneracy in its reverse-translated sequence (SEQ ID NO:4; Drupe20). The Drupe20 primer (i) is the 512-fold degenerate nucleotide sequence corresponding to the amino acid sequence presented as SEQ ID NO:3, and (ii) was used as the 3'-primer.

The 5' PCR primer (DrupeRAN18, SEQ ID NO:5, corresponding to the cDNA primer, dRANDOM) was designed for the 3' end. Polymerase chain reaction (PCR; Perkin-Elmer Cetus, Norwalk, CT; Mullis, 1987; Mullis, *et al.*, 1987, was performed following the manufacturer's procedure using "AMPLITAQ" (Perkin Elmer Cetus), PCR buffer II (50.0 mM KCl, 10 mM Tris-HCl, pH 8.3), 2 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, mature green drupelet cDNA and Drupe20 and DrupeRAN18 primers under the following conditions:

1 cycle at 95°C, 1 minute, 35 cycles at 95°C for 1 minute, 42°C for 1 minute and 72°C for 1 minute, 1 cycle at 72°C for 5 minutes, and cooling to 5°C.

There were two major products of the amplification reaction: a predominant product of approximately 700 bp and a less abundant product of approximately 500 bp. The 700 bp band was isolated from a 1% "SEAPLAQUE" agarose gel using  $\beta$ -agarase (NEB, Beverly, MA) according to the supplier's instructions. This fragment was then ligated to the vector pCRII, the TA cloning vector from Invitrogen (San Diego, CA), following the manufacturer's instructions.

The cDNA clones of the *dru1* gene were identified by screening plasmid miniprep DNA prepared from 1.6 ml of culture using the alkaline lysis method (Ausubel, *et al.*, 1992). The double-stranded DNA was sequenced by the dideoxy chain-termination method using the "SEQUENASE" ver.2 enzyme and kit components (United States Biochemical, Cleveland, Ohio) and [ $\alpha$ -<sup>35</sup>S]-dATP (DuPont/NEN). The reactions were primed with the M13 universal forward and reverse primers (NEB, Beverly, MA). Sequencing reactions were resolved on an acrylamide gel ("LONG RANGER GEL," FMC, Rockland, Maine) and bands detected by autoradiography.

The sequence was read from the autoradiograph and analyzed for its homology with the reverse translated N-terminal protein sequence from *drupe1*. The actual DNA sequence was determined, as opposed to the degenerate DNA sequence obtained through reverse translation of the protein sequence. In addition, the correlation between the cDNA and the remainder of the N-terminal protein sequence was confirmed. A clone (designated pAG-301) was selected, following these criteria, for further characterization. The nucleic acid sequence of the *dru1* cDNA insert of pAG-301 is presented as SEQ ID NO:10.

The entire *dru1* cloning procedure from cDNA synthesis to inverse PCR of a genomic copy of the gene is shown schematically in Figures 2A and 2B.

#### EXAMPLE 4

##### Recovering the Genomic DNA Fragment Corresponding to the *dru1* cDNA

The "CTAB" (hexadecyl-trimethyl-ammonium bromide) method (Doyle and Doyle, 1990) was used to extract DNA from raspberry leaves. PCR primers (DruGen5', SEQ ID NO:6; DruGen3', SEQ ID NO:7) were designed based upon the complete *dru1* cDNA sequence. "OLIGO," a multi-functional program from National Biosciences, Inc. (Plymouth, MN), was used to facilitate design of the primers. PCR was performed following the manufacturer's procedure using "AMPLITAQ" (Perkin-Elmer Cetus), PCR buffer (50.0 mM KCl, 10 mM Tris-HCl pH 8.3, and 1.5 mM MgCl<sub>2</sub>), 0.2 mM of each dNTP, raspberry genomic DNA and DruGen5' and DruGen3' primers under the following ("HOT START") conditions:

- 1 cycle of 97°C for 5 minutes, after which the "AMPLITAQ" was added,
- 2 cycles of 97°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute,
- 25 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute,

1 cycle of 72°C for 5 minutes, and cooling to 5°C.

This amplification reaction produced 3 major products: a predominant product of 710 bp and 2 less abundant products of 690 and 625 bp. The PCR reaction products were then ligated to the vector pCRII, the TA cloning vector from Invitrogen (San Diego, CA), following the  
5 manufacturer's instructions. A clone was selected with a 710 bp insert and designated pAG-302.

Plasmid DNA of pAG-302 was prepared from 1.6 ml of culture using the alkaline lysis method (Ausubel, *et al.*, 1992) and sequenced by the dideoxy chain-termination method using "SEQUENASE" ver.2 enzyme and kit components (USB, Cleveland, Ohio) and [ $\alpha$ -35S]-dATP (DuPont/NEN). The sequencing reactions were primed with the M13 universal forward and  
10 reverse primers (NEB, Beverly, MA). Further sequencing reactions were primed with 2 additional internal primers. Sequencing reactions were resolved on an acrylamide gel and detected through autoradiography.

The sequence of the *druI* genomic DNA insert in pAG-302 is presented as SEQ ID NO:11.

The sequence of the clone demonstrated that a genomic DNA fragment corresponding to the  
15 *druI* cDNA had been isolated.

#### EXAMPLE 5

##### Recovering the 5' Flanking Region of the *druI* Genomic DNA Through Inverse PCR

Inverse PCR primers (designated DruInvUp, SEQ ID NO:8, and DruInvLow, SEQ ID NO:9) were designed based upon the genomic DNA sequence and optimized using OLIGO. Genomic  
20 raspberry DNA was digested with restriction enzyme *NsiI*. *NsiI* was chosen because, based on the cDNA sequence, *NsiI* was known to cut in the 3'-untranslated region of the gene. A small portion of the *NsiI* digested DNA was run on an analytical agarose gel and a Southern transfer was performed (Ausubel, *et al.*, 1992).

The Southern blot was probed with the cDNA fragment contained in pAG-302. The probe  
25 identified a *NsiI* fragment of about 2-2.3 kb: this fragment hybridized strongly with the genomic clone. A second, smaller fragment hybridized to the probe as well but hybridized weakly with the genomic clone.

The remaining *NsiI*-digested raspberry DNA was electrophoresed on a 1% "SEAPLAQUE" agarose gel (FMC, Rockland, ME). Using a *BstEII* lambda size standard as a guide, the digested  
30 DNA in the range of 2-2.3 kb was excised from the gel. The DNA was purified using  $\beta$ -agarase (NEB, Beverly, MA) following the manufacturer's instructions. The DNA was self ligated at a relatively dilute concentration (1  $\mu$ g/ml) to bias the formation of circular ligation reaction products (Ochman, *et al.*, 1990).



Inverse PCR was subsequently performed on the self-ligated, *Nsi*I-digested, size-selected, genomic raspberry DNA. "AMPLITAQ" from Perkin Elmer Cetus was used to amplify the DNA. The manufacturer's procedure was followed using PCR buffer, 0.2 mM of each dNTP, raspberry genomic DNA (prepared as described herein), and *Dru*InvUp and *Dru*InvLow primers. The following ("HOT START") reaction conditions were employed:

One cycle at 97°C for 5 minutes, after which the "AMPLITAQ" was added,  
2 cycles at 97°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute,  
25 cycles at 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute,  
1 cycle at 72°C for 5 minutes, and cooling to 5°C.

This reaction produced 2 major amplification products, one of 1.8 kb and one of 900 bp. The 1.8 kb band was isolated from a 1% "SEAPLAQUE" agarose gel using  $\beta$ -agarase. This fragment was ligated to pCRII to give rise to pAG-310.

The pAG-310 insert was sequenced in its entirety (SEQ ID NO:12) and the *dru*1 insert sequence was found to be identical to the cDNA clone (SEQ ID NO:10) and the genomic clone (SEQ ID NO:11) in the regions where sequence was shared. The normal elements of plant genes and their regulatory components were identified (Figures 3A and 3B) including a CAAT box, TATA box, ATG start codon, two exons, an intron, splicing sites, a stop codon and polyadenylation sites.

The gene organization and protein structure of *dru*1 is schematically displayed in Figure 4. The gene encodes a protein having the predicted amino acid sequence presented as SEQ ID NO:13. The predicted protein has a calculated molecular weight of 17,087.64 and an estimated pI of 4.80. A Kyte-Doolittle hydrophobicity plot of the *dru*1 protein is presented as Figure 5.

#### EXAMPLE 6

##### Characterization of *dru*1 Gene Expression

##### A. RNA DOT BLOTS.

RNA dot blots were prepared using 5  $\mu$ g of total raspberry leaf RNA and 5  $\mu$ g each of total receptacle RNA from green, mature green, breaker & orange/ripe raspberries (corresponding to stages I, II, III, IV, respectively, in Figure 6). The blots were probed with the *dru*1 cDNA fragment, labeled with [32-P]dCTP (> 3000 Ci/mmol) by the random primed method (Boehringer Mannheim Biochemicals, Random Primed reaction kit, Indianapolis, IN).

The blots were allowed to hybridize overnight at 45°C in "HYBRISOL I" (Oncor, Gaithersburg, MD). A probe concentration of  $1.2 \times 10^7$  DPM/ml was used. The blot was washed after the overnight hybridization with a final wash using  $0.1 \times$  SSC at 42°C for 1 hour.

The hybridizing probe was detected through standard autoradiographic methods. The exposure of the blot to film was for 4 hours and 10 minutes with an intensifying screen at -80°C.

The results of this analysis are shown in Figure 6. In the figure the RNA dots are, respectively from left to right, leaf RNA and receptacle RNA from green (Figure 6, "I"), mature green (Figure 6, "II"), breaker (Figure 6, "III") and orange/ripe raspberries (Figure 6, "IV").

#### B. FURTHER RNA HYBRIDIZATION ANALYSIS.

A plant RNA extraction method (Chang, *et al.*, 1993) was used for receptacles and leaves. The raspberry drupelet RNA extraction method described above was used for the drupelets and raspberry fruit.

A Northern blot was prepared using 5 µg/lane of each sample RNA. The RNA samples were as follows: raspberry leaf (Figure 7, lane 1), mature green raspberry receptacles (Figure 7, lane 2), orange/ripe raspberry receptacles (Figure 7, lane 3), mature green raspberry drupelets (Figure 7, lane 4), and orange/ripe raspberry drupelets (Figure 7, lane 5).

The blot was probed with the *drul* cDNA fragment, labeled with [<sup>32</sup>P]dCTP (>3000 Ci/mmmole) by random primed reactions. Hybridization was carried out overnight at 45°C in "HYBRISOL I" (Oncor, Gaithersburg, MD). A probe concentration of  $4.2 \times 10^6$  DPM/ml was used. The blot was washed after the overnight hybridization with a final wash using  $0.1 \times$  SSC at 50°C for 30 minutes. The hybridizing probe was detected through standard autoradiographic methods. The exposure of the blot to film was for 1 hour at room temperature without an intensifying screen.

The results of this analysis are presented in Figure 7 and support a stage specific expression pattern in drupelets.

#### C. PROTEIN EXPRESSION ANALYSIS.

Protein lysates were prepared (as described in Example 1) from raspberry drupelets at various stages of ripening. The lysates were size-fractionated by PAGE and the gel stained with Coomassie blue (50% MeOH, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>). The results of this work are presented in Figure 8. In the figure the lysates in the lanes were as follows: lane 1, green drupelet; lane 2, mature green drupelet; lane 3, breaker drupelet; lane 4, orange drupelet; and lane 5, ripe drupelet. The results of this analysis supports a stage specific expression pattern in drupelets.

## EXAMPLE 7

Chimeric Genes Containing the *dru1* Promoter

## A. CONSTRUCTION OF A DRU1PRO:SAMASE BINARY VECTOR.

A fragment containing the *Dru1* promoter was PCR amplified from pAG-310 using primers  
5 *DruPro5'RI* (SEQ ID NO:14) and *DruPro3'* (SEQ ID NO:15) and standard PCR reaction conditions. The amplification reaction produced a 1.3 kb fragment product. This fragment was digested to completion with *EcoRI* and *NcoI*. The digested fragment was ligated into pAG-112, a pUC vector carrying an *AdoMetase* encoding gene (Ferro, *et al.*, 1995) with a *nos* terminator. The resulting plasmid was designated pAG-119.

10 pAG-119 plasmid DNA was digested to completion with *SmaI* and *HindIII*. A 2.1 kb fragment containing *Dru1pro/SAM-Kozak/Nos* terminator was recovered from 1% "SEA-PLAQUE" agarose using  $\beta$ -agarase. pAG-4000 was obtained from pPZP-200 (Hajdukiewicz, *et al.*, 1994) by inserting a CMVV/*nptII/G7* terminator gene cassette into the multiple cloning site of pPZP-200. The CMVV (Cassava mottle vein virus) promoter was obtained from Scripps  
15 Research Institute, La Jolla, CA). pAG-4000 was digested with *SmaI* and *HindIII* and ligated to the 2.1kb pAG-119 fragment to form vector pAG-4032. The details of this construction are described schematically in Figure 9.

The complete nucleotide sequence of the *dru1* promoter:SAMase chimeric gene is presented as SEQ ID NO:16. The predicted amino acid coding sequence is presented as SEQ ID NO:17.

## 20 B. CONSTRUCTION OF A DRU1PRO:PGIP BINARY VECTOR.

The PGIP gene (Toubart, *et al.*, 1992) and its 3' untranslated region (UTR) was PCR amplified from pAD-16 (Toubart, *et al.*, 1992) using the primers PGIPNco5' (SEQ ID NO:18) and PGIPPst3' (SEQ ID NO:19). The amplification reaction produced a product of 1.8 kb. This 1.8 kb fragment included a portion of the cloning vector. The fragment was digested with *NcoI*  
25 and *PstI* to completion resulting in a 1290 bp fragment which no longer contained portions of the cloning vector.

pAG-119 (see above) was prepared by digestion to completion with *NcoI* and *PstI*. This removed the *SamK* portion of the plasmid. The remaining portion of the plasmid was then ligated to the PGIP-containing fragment described above. This new plasmid was designated pAG-129.

30 pAG-129 was digested to completion with *XbaI* and *PvuII* (a restriction enzyme whose cleavage results in blunt ends). The 2.87 kb fragment containing *Dru1pro/PGIP/Nos* terminator was recovered from 1% "SEAPLAQUE" agarose by using  $\beta$ -agarase. The vector pAG-4033 was prepared by digestion to completion with *XbaI* and *SmaI* (a restriction enzyme whose cleavage results in blunt ends). This digestion removed the *Dru1pro/SAM-Kozak/Nos* terminator portion

of the plasmid. The remaining portion of the plasmid was then ligated to the *DruI*pro/PGIP/Nos terminator fragment described above. This new plasmid was named pAG-4033 and its construction is described schematically in Figure 10.

The complete nucleotide sequence of the *druI* promoter:PGIP chimeric gene is presented as  
5 SEQ ID NO:20. The predicted amino acid coding sequence is presented as SEQ ID NO:21.

#### EXAMPLE 8

##### Southern Blot Analysis of *druI* Homologues in Several Species of Plants

A Southern blot analysis is conducted to determine if sequences homologous to the raspberry  
*druI* gene are present in other plant species. The blot consists of *HindIII* digests of six genomic  
10 plant DNAs, for example, tomato, raspberry, strawberry, plum, cherry and peach, along with size  
standards. Probes can be constructed using *druI* coding sequence-specific primers and polymerase  
chain reaction (PCR; Mullis, 1987; Mullis, *et al.*, 1987). Alternatively, the 700 base pair insert  
from pAG-301 (SEQ ID NO:10) is isolated by digestion with *EcoRI* followed by size fractionation.  
The DNA fragment is then radioactively-labeled using the Bohringer Mannheim Biochemical (Indi-  
15 anapolis, IN) "RANDOM PRIMED DNA LABELING" kit. The blot is hybridized with the *druI*-  
specific probe following standard methods (Maniatis, *et al.*, 1982). Exemplary hybridization  
conditions are as follows: the blot is hybridized overnight at 45°C with the *druI* probe in "HY-  
BRISOL I" hybridization cocktail (Oncor, Gaithersburg, MD). The final (most stringent) wash  
is 0.1% SSC, 0.1% SDS for 30 minutes at room temperature (22°C).

20 An autoradiograph of the blot is used to identify plant species to whose genomic DNA the  
*druI* probe can hybridize.

#### EXAMPLE 9

##### Isolation of DNA Fragments Homologous to *druI* from a Strawberry Genomic Library

###### A. Screening of the Library.

25 A custom strawberry genomic library in lambda GEM-11 is obtained from Novagen  
(Madison, WI) and screened by standard methods with the *druI* gene probe described above.  
Lambda clones which hybridized to the probe are identified. The clones are purified by 3 rounds  
of plaque purification. Hybridization-positive clones are selected for further analysis.

###### B. Analysis of a Positive Clone.

30 A clone of interest is digested with several enzymes (*e.g.*, *Apa* I, *Bam* HI, *Eco* RI, *Hind* III,  
*Nco* I, *Sac* I, and *Sal* I), run on a gel, and transferred to a "SUREBLOT" nylon membrane  
(Oncor, Gaithersburg, MD). The blot is hybridized overnight at 45°C with the *druI* probe in  
"HYBRISOL I" hybridization cocktail (Oncor, Gaithersburg, MD). The final (most stringent)  
wash is 0.1% SSC, 0.1% SDS for 30 minutes at room temperature (22°C).

A hybridization-positive fragment is subcloned into pGEM5Zf(+) (Promega, Madison, WI) and further characterized. The nucleic acid sequence of the insert is determined and the amino acid sequence predicted from the nucleic acid sequence. These sequences are then compared to the raspberry *dru1* nucleic acid and protein sequences.

- 5 Additional strawberry *dru1* gene sequences are obtained by further hybridization screening of strawberry genomic library clones.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Agritope, Inc.
- (ii) TITLE OF INVENTION: PLANT TISSUE-SPECIFIC PROMOTERS FOR  
REGULATED EXPRESSION OF TRANSGENES IN PLANTS
- 10 (iii) NUMBER OF SEQUENCES: 22
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: Dehlinger & Associates  
(B) STREET: 350 Cambridge Avenue, Suite 250  
(C) CITY: Palo Alto  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) ZIP: 94306
- 20 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- 30 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: US 08/592,936  
(B) FILING DATE: 29-JAN-1996
- (viii) ATTORNEY/AGENT INFORMATION:
- 35 (A) NAME: Evans, Susan T.  
(B) REGISTRATION NUMBER: 38,443  
(C) REFERENCE/DOCKET NUMBER: 4257-0012.41
- (ix) TELECOMMUNICATION INFORMATION:
- 40 (A) TELEPHONE: (415) 324-0880  
(B) TELEFAX: (415) 324-0960

## (2) INFORMATION FOR SEQ ID NO:1:

30

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

10 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: amino terminal drupel sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15

Val Leu Gln Gly Lys Val Glu Ala Asp Ile Glu Ile Ser Ala Pro Ala  
1 5 10 15

Ala Lys Phe Tyr Asn Leu Phe Lys Ser Glu Ala Xaa Trp Val  
20 25 30

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

35

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: dRANDOM primer

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TAGGCTCGTA GACTCTTTTT TTTTTTTTTT

30

## (2) INFORMATION FOR SEQ ID NO:3:

45

31

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

10

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: drul partial amino acid sequence

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gln Gly Lys Val Glu Ala Asp  
1 5

20

(2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

35

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: reverse translated sequence of SEQ  
ID NO:3

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CARGGNAARG TNGARCGNGA

20

(2) INFORMATION FOR SEQ ID NO:5:

45



32

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

5 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

10

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: DrupeRAN18 primer

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAGGCTCGTA GACTCTTT

20

18

(2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

25 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

35 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: DruGen 5' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

40

AAGGTGGAGG CTGACATT

18

(2) INFORMATION FOR SEQ ID NO:7:

45 (i) SEQUENCE CHARACTERISTICS:

33

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

10

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: DruGen 3' primer

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGACGGTAT TAGTGCATAA CA

22

20(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

35

(C) INDIVIDUAL ISOLATE: DruInvUp primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

40TGAATGGGTT GGAAGGAGAT GTGT

24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs

45

34

(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: DruInvLow primer

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGGTGCCAG TTTGAGAAGT TTTG

24

(2) INFORMATION FOR SEQ ID NO:10:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 751 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

25

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

30

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pag301 insert, drul cDNA clone

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGGAAAGG TGGAGGCTGA CATTGAAATC TCAGCACCTG CTGACAAGTT CTACAACCTC

60

40

TTCAAGAGTG AGGCTCACCA CGTCCCCAAA ACTTCTCAAA CTGGCACCAT AACCGGAGTT

120

GCGGTGCATG AAGGAGACTG GGAAACTGAT GGCTCCATTA AGATTTGGAA TTATGCAATA

180

45GAGGGCGAAG TGGGAACATT CAAGGAGAAA GTAGAGCTAG ACGATGTGAA CAAGGCAATA

240

35

ATTCTGAATG GGTGGAAGG AGATGTGTTT CAGTATTACA AGAGCTTCAA GCCCGTCTAT 300  
CAATTCACCTC AAAAGAATGA TGGCAGCAGC ATTGCCAAAG TGTCCATTGA ATATGAGAAA 360  
5CTGAGTGAGG AAGTTGCAGA TCCAAATAAG TACATTCGCT TGATGACTAA TATCGTCAAG 420  
GATCTTGATG CCCACTTCAT CAAGGCATAA AAGGGATATT ATAATAAATC AAGCATATGA 480  
AACACGATGA AAAGAGAGCT AGCCACTATC TACTGCTGGT TTATAAGTTT AAAGATAATC 540  
10 ATGTGAACGT TGTAATGCAT GCTTTGTTTG GTTACTTCGT TTAATGTCT TGTTATGCAC 600  
TAATACCGTC AGTGTAAATAA AAGCTAGTGT GAAAGGATCT GATATATTGT GATGTATCAT 660  
15GTATTCAACT ACCAACTATA TATGGTATCA TATTTATATA TCAAATAAAT TAATGTGAAA 720  
AAAAAAAAA AAAAAAAGAG TCTACGAGCC T 751

## (2) INFORMATION FOR SEQ ID NO:11:

20

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 745 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

25

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

30

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pag302, drul genomic clone

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGGTGGAGG CTGACATTGA AATCTCAGCA CCTGCTGACA AGTTCTACAA CCTCTTCAAG 60  
40 AGTGAGGCTC ACCACGTCCC CAAACTTCT CAAACTGGCA CCATAACCGG AGTTGCGGTG 120  
CATGAAGGAG ACTGGGAAAC TGATGGCTCC ATTAAGATTG GGAATTATGC AATAGGTAAG 180  
45CCATTATGTT GTTAGATTGT TAATTTAGAT TATTAACCAA AGCTGGCTTT GAATCACTAC 240

AATATATATT AGGGCACGCC AGTACAGATT TTCTGTTTAT AATTGTTTCA GTGATTATTT 300  
TCTTACAAAT ATAGAGGGCG AAGTGGGAAC ATTCAAGGAG AAAGTAGAGC TAGACGATGT 360  
5GAACAAGGCA ATAATTCTGA ATGGGTTGGA AGGAGATGTG TTCCAGTATT ACAAGAGCTT 420  
CAAGCCCGTC TATCAATTCA CTCAAAAGAA TGATGGCAGC AGCATTGCCA AAGTGTCCAT 480  
TGAATATGAG AACTGAGTG AGGAAGTTGC AGATCCAAAT AAGTACATTC GCTTGATGAC 540  
10 TAATATCGTC AAGGATCTTG ATGCCCACTT CATCAAGGCA TAAAAGGGAT ATTATAATAA 600  
ATCAAGCATA TGAAACACGA TGAAAAGAGA GCTAGCCACT ATCTACTGCT GGTTTATAAG 660  
15TTTAAAGATA ATCATGTGAA CGTTGTAATG CATGCTTTGT TTGGTTACTT CGTTTTAATG 720  
TCTTGTTATG CACTAATACC GTCAG 745

(2) INFORMATION FOR SEQ ID NO:12:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2213 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

30 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: PAG310 insert sequence

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGCATATCA ACAACTACGA ATAAAGAGAT CAGCCTTTCC GTATCTGGTG GATGTTTGAG 60  
40TCGGTGATGA CCATCTAATT AAAGAAAGAA GAAAAATTAT ACATATTGTG GACCTCCCCA 120  
TATATAATTC TTATCATCTT TGTTACTGCC ATTATGATTA TAAATGATA TTAAAGGGAT 180  
GGTGTACCGT GTACTAATCA AATATCTACC TGATCTTATT GATTGAAAG ATCATAAAAA 240  
45

	GAAATTAAAA TTGTTCAAAA TAAACCCCTA GAATTATATA TAGTTCATTA AGTTCAAATT	300
	AATTCGTTTG AAACGTGTTA AGCAACCCTA CAACGTACTA AGCACCCTAG CTCCCTTTGC	360
	5CTCTCGGCGG TAAGAGGAGA TATCCTCAGT CGAATTATGA GCCGATCGAG GAAAGCTCGA	420
	TCAGTTGGAA AATCTTTCTT TCTTATGGCC AAGTTGTTTC AAACAATATA TTGAATTATT	480
	GACTCTTAGC AACTTAAGTT TCAAACCGTG ACGAACCAAT AAAATTTGAC AAATTAATCA	540
10	CTTTAAGTGC CTAGTGGATC AGCGTCTAGG TTGGGAACCC CTCTACCTGC GTTTGATTCA	600
	CCAAGCTATC AAAATGGTCA GACACTGTGC TGCAATGCAC AATTGGAGCA TTTCACATGC	660
	15GTTGCATGAA TTATTCCTTG GGTTAGGAAA CCTTTGAAAT ACCTTGACTA AGGTAAAAAA	720
	AAAACTTGA CAAATTAATA AATATTAATA TTGATTTTGT ACGTACACGA CTTAACCAAA	780
	CTCTCAATGA TTTATTGATT TCTAATATAT ATATTAATAA CGTACGTCTA ATTGGATCAT	840
20	TCATGATCTA CAGCCATCAC ATCTCAGATG ATTTTCTTGC AATGAATTGC CTAAGCTGGC	900
	GTTATTATCT TTTTTTCATA ATACAGTTT AAAAAGGGT ACGTATTGGA GCTGGTGATG	960
	25ACTTCTTAAG AAACAACAAA TTAACGCCAT AGCTATTTGA TTTATATATC CAAAAGGAGA	1020
	AAATGTATAA GATCGTTGCT TACTTAATT GCAGGCTAGG TTAATTGACA TCAAATAATT	1080
	GAAGAGTACG TAGGGCCAAT GTTGCTGAGA TCTAGCATCA ATAATAGGAT TTGGCTTGTC	1140
30	GATCGATCAT CTTTATTTAA TTGAGAGGTA TGTATCCATA TGTTTTCTGA AATTAAATA	1200
	TTACCTAATA ATTGAGCTGA AACTGTAGTG AATTTAACCT TTTCTAAGTT CTGCCCATAT	1260
	35ATAACATACC ACATAGGTAG CTGATCGATC GATCATATAT ATGTACTTAG GGTTCGTATC	1320
	AGTATCAATA TCGATCACAA GTGCTGATAA TTAACATGG TTCTTCAAGG TAAGGTGGAG	1380
	GCTGACATTG AAATCTCAGC ACCTGCTGAC AAGTTCTACA ACCTCTTCAA GAGTGAGGCT	1440
40	CACCACGTCC CCAAACTTC TCAAACCTGGC ACCATAACCG GAGTTGCGGT GCATGAAGGA	1500
	GACTGGGAAA CTGATGGCTC CATTAGATT TGAATTATG CAATAGGTAA GCCATTATGT	1560
	45TGTTAGATTG TTAATTTAGA TTATTAACCA AAGCTGGCTT TGAATCACTA CAATATATAT	1620

TAGGGCACGC CAGTACAGAT TTTCTGTTTA TAATTGTTTC AGTGATTATT TTCTTACAAA 1680  
TATAGAGGGC GAAGTGGGAA CATTCAAGGA GAAAGTAGAG CTAGACGATG TGAACAAGGC 1740  
5AATAATTCTG AATGGGTGG AAGGAGATGT GTTCCAGTAT TACAAGAGCT TCAAGCCCGT 1800  
CTATCAATTC ACTCAAAGA ATGATGGCAG CAGCATTGCC AAAGTGTCCA TTGAATATGA 1860  
GAACTGAGT GAGGAAGTTG CAGATCCAAA TAAGTACATT CGCTTGATGA CTAATATCGT 1920  
10 CAAGGATCTT GATGCCCCACT TCATCAAGGC ATAAAAGGGA TATTATAATA AATCAAGCAT 1980  
ATGAAACACG ATGAAAAGAG AGCTAGCCAC TATCTACTGC TGGTTTATAA GTTTAAAGAT 2040  
15AATCATGTGA ACGTTGTAAT GCATGCTTTG TTTGGTTACT TCGTTTTAAT GTCTTGTTAT 2100  
GCACTAATAC CGTCAGTGTA ATAAAAGCTA GTGTGAAAGG ATCTGATATA TTGTGATGTA 2160  
TCATGTATTC AACTACCAAC TATATATGGT ATCATATTTA TATATCAAAT AAA 2213  
20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 152 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- 35 (C) INDIVIDUAL ISOLATE: predicted amino acid coding sequence  
of drul

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

40 Met Val Leu Gln Gly Lys Val Glu Ala Asp Ile Glu Ile Ser Ala Pro  
1 5 10 15  
Ala Asp Lys Ph Tyr Asn Leu Phe Lys Ser Glu Ala His His Val Pro  
45 20 25 30

39

Lys Thr Ser Gln Thr Gly Thr Ile Thr Gly Val Ala Val His Glu Gly  
 35 40 45  
 Asp Trp Glu Thr Asp Gly Ser Ile Lys Ile Trp Asn Tyr Ala Ile Glu  
 5 50 55 60  
 Gly Glu Val Gly Thr Phe Lys Glu Lys Val Glu Leu Asp Asp Val Asn  
 65 70 75 80  
 Lys Ala Ile Ile Leu Asn Gly Leu Glu Gly Asp Val Phe Gln Tyr Tyr  
 85 90 95  
 Lys Ser Phe Lys Pro Val Tyr Gln Phe Thr Gln Lys Asn Asp Gly Ser  
 100 105 110  
 Ser Ile Ala Lys Val Ser Ile Glu Tyr Glu Lys Leu Ser Glu Glu Val  
 115 120 125  
 Ala Asp Pro Asn Lys Tyr Ile Arg Leu Met Thr Asn Ile Val Lys Asp  
 130 135 140  
 Leu Asp Ala His Phe Ile Lys Ala  
 145 150

## 25(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 29 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA  
 (iii) HYPOTHETICAL: NO  
 (iv) ANTI-SENSE: NO  
 (vi) ORIGINAL SOURCE:  
 (C) INDIVIDUAL ISOLATE: DruPro5'RI primer  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

45GAGAATTCCC CGGGCAGATC AACAACTAC



40

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

10

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## 15 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: DruPro3' primer

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

20

GCGCGGCCAT GGTTAATTAT CAG

23

## (2) INFORMATION FOR SEQ ID NO:16:

## 25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2145 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
30 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (iii) HYPOTHETICAL: NO

## 35 (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: drul promoter:SAMase chimeric gene

40

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCCGGGCAGA TCAACAATA CGAATAAAGA GATCAGCCTT TCCGTATCTG GTGGATGTTT

60

45GAGTCGGTGA TGACCATCTA ATTAAAGAAA GAAGAAAAAT TATACATATT GTGGACCTCC

120

	CCATATATAA TTCTTATCAT CTTTGTTACT GCCATTATGA TTATAAAATG ATATTAAAGG	180
	GATGGTGTAC CGTGTACTAA TCAAATATCT ACCTGATCTT ATTGATTGA AAGATCATAA	240
	5AAAGAAATTA AAATTGTTCA AAATAAACCC CTAGAATTAT ATATAGTTCA TTAAGTTCAA	300
	ATTAATTCGT TTGAAACGTG TTAAGCAACC CTACAACGTA CTAAGCACCC TAGCTCCCTT	360
	TGCCTCTCGG CGGTAAGAGG AGATATCCTC AGTCGAATTA TGAGCCGATC GAGGAAAGCT	420
10	CGATCAGTTG GAAAATCTTT CTTTCTTATG GCCAAGTTGT TTCAAACAAT ATATTGAATT	480
	ATTGACTCTT AGCAACTTAA GTTTCAAACC GTGACGAACC AATAAAATTT GACAAATTAA	540
	15TCACTTTAAG TGCCTAGTGG ATCAGCGTCT AGGTTGGGAA CCCCTCTACC TGC GTTTGAT	600
	TCACCAAGCT ATCAAAATGG TCAGACACTG TGCTGCAATG CACAATTGGA GCATTTTACA	660
	TGCGTTGCAT GAATTATTCC TTGGGTTAGG AAACCTTTGA AATACCTGA CTAAGGTAAA	720
20	AAAAAAACT TGACAAATTA ATAAATATTA ATATTGATTT TGTACGTACA CGACTTAACC	780
	AAACTCTCAA TGATTTATTG ATTTCTAATA TATATATTAA TAACGTACGT CTAATTGGAT	840
	25CATTCAATGAT CTACAGCCAT CACATCTCAG ATGATTTTCT TGCAATGAAT TGCCTAAGCT	900
	GGCGTTATTA TCTTTTTTTC ATAATACAGT TTTAAAAAAG GGTACGTATT GGAGCTGGTG	960
	ATGACTTCTT AAGAAACAAC AAATTAACGC CATAGCTATT TGATTTATAT ATCCAAAAGG	1020
30	AGAAAATGTA TAAGATCGTT GCTTACTTAA TTTGCAGGCT AGGTTAATTG ACATCAAATA	1080
	ATTGAAGAGT ACGTAGGGCC AATGTTGCTG AGATCTAGCA TCAATAATAG GATTTGGCTT	1140
	35GTCGATCGAT CATCTTTATT TAATTGAGAG GTATGTATCC ATATGTTTTT TGAAATTAAA	1200
	ATATTACCTA ATAATTGAGC TGAAACTGTA GTGAATTTAA CCTTTTCTAA GTTCTGCCCCA	1260
	TATATAACAT ACCACATAGG TAGCTGATCG ATCGATCATA TATATGTACT TAGGGTTCTG	1320
40	ATCAGTATCA ATATCGATCA CAAGTGCTGA TAATTAACCA TGGTTTTTAC TAAAGAGCCT	1380
	GCGAACGTCT TCTATGTACT GGTTCCTGCT TTCCGTTCTA ACCTCTGCGA TGAGGTGAAT	1440
	45ATGAGCAGAC ACCGCCACAT GGTAAGCACT TTACGTGCCG CACCGGGTCT TTATGGCTCC	1500

GTTGAGTCAA CCGATTTGAC CGGGTGCTAT CGTGAGGCAA TCTCAAGCGC ACCAACTGAG 1560  
 GAAAAAACTG TTCGTGTACG CTACAAGGAC AAAGCGCAGC CACTCAATGT TGCACGCCTA 1620  
 5GCTTCTAATG AGTGGGAGCA AGATTGCGTA CTGGTATACA AATCACAGAC TCACACGGCT 1680  
 GGTCTGGTGT ACGCTAAAGG TATCGACGGG TATAAGGCTG AACGTCTGCC GGGTAGTTTC 1740  
 CAAGAGGTTT CTAAAGGCGC ACCGCTGCAA GGCTGCTTCA CTATTGATGA GTTCGGTTCG 1800  
 10 CGCTGGCAAG TACAATAAGT GTTAACTCA AGGTCATGCA CGATGCGTGG CGGATCGGGT 1860  
 ACCGAGCTCG AATTTGACC TGCAGATCGT TCAAACATTT GGCAATAAAG TTTCTTAAGA 1920  
 15 TTGAATCCTG TTGCCGGTCT TGCGATGATT ATCATATAAT TTCTGTTGAA TTACGTTAAG 1980  
 CATGTAATAA TTAACATGTA ATGCATGACG TTATTTATGA GATGGGTTTT TATGATTAGA 2040  
 GTCCCGCAAT TATACATTTA ATACGCGATA GAAAACAAAA TATAGCGCGC AAAC TAGGAT 2100  
 20 AAATTATCGC GCGCGGTGTC ATCTATGTTA CTAGATCTTC TAGAA 2145

## (2) INFORMATION FOR SEQ ID NO:17:

## 25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 152 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## 30 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

35 (C) INDIVIDUAL ISOLATE: predicted amino acid coding sequence  
 of SEQ ID NO:16

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

40

Met Val Phe Thr Lys Glu Pro Ala Asn Val Phe Tyr Val Leu Val Ser  
 1 5 10 15

45

Ala Phe Arg Ser Asn Leu Cys Asp Glu Val Asn Met Ser Arg His Arg  
 20 25 30

43

His Met Val Ser Thr Leu Arg Ala Ala Pro Gly Leu Tyr Gly Ser Val  
 35 40 45  
 Glu Ser Thr Asp Leu Thr Gly Cys Tyr Arg Glu Ala Ile Ser Ser Ala  
 5 50 55 60  
 Pro Thr Glu Glu Lys Thr Val Arg Val Arg Tyr Lys Asp Lys Ala Gln  
 65 70 75 80  
 10 Pro Leu Asn Val Ala Arg Leu Ala Ser Asn Glu Trp Glu Gln Asp Cys  
 85 90 95  
 Val Leu Val Tyr Lys Ser Gln Thr His Thr Ala Gly Leu Val Tyr Ala  
 100 105 110  
 15 Lys Gly Ile Asp Gly Tyr Lys Ala Glu Arg Leu Pro Gly Ser Phe Gln  
 115 120 125  
 Glu Val Pro Lys Gly Ala Pro Leu Gln Gly Cys Phe Thr Ile Asp Glu  
 20 130 135 140  
 Phe Gly Arg Arg Trp Gln Val Gln  
 145 150

## 25(2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs  
 (B) TYPE: nucleic acid  
 30 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## 35 (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- 40 (C) INDIVIDUAL ISOLATE: PGIP Nco5' Primer

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

45GGGGCTCCAT GGCTCATT

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

10

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## 15 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: PGIP Pst3' Primer

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

20

GGGCGAAAAA CCGTCTATCA G

21

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 2917 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

## 30 (ii) MOLECULE TYPE: DNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

35

## (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: sequence of the drul:PGIP chimeric  
gene

40

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAATTCCCCG GGCAGATCAA CAACTACGAA TAAAGAGATC AGCCTTTCCG TATCTGGTGG

60

45ATGTTTGAGT CCGTGATGAC CATCTAATTA AAGAAAGAAG AAAAATTATA CATATTGTGG

120

ACCTCCCAT ATATAATTCT TATCATCTTT GTTACTGCCA TTATGATTAT AAAATGATAT 180  
TAAAGGGATG GTGTACCGTG TACTAATCAA ATATCTACCT GATCTTATTG ATTTGAAAGA 240  
5TCATAAAAAG AAATTAAAAT TGTTCAAAAT AAACCCCTAG AATTATATAT AGTTCATTAA 300  
GTTCAAATTA ATTGTTTGA AACGTGTAA GCAACCCTAC AACGTACTAA GCACCCTAGC 360  
TCCCTTTGCC TCTCGGCGGT AAGAGGAGAT ATCCTCAGTC GAATTATGAG CCGATCGAGG 420  
10 AAAGCTCGAT CAGTTGAAA ATCTTTCTTT CTTATGGCCA AGTTGTTCA AACAATATAT 480  
TGAATTATTG ACTCTTAGCA ACTTAAGTTT CAAACCGTGA CGAACCAATA AAATTTGACA 540  
15AATTAATCAC TTTAAGTGCC TAGTGGATCA GCGTCTAGGT TGGGAACCCC TCTACCTGCG 600  
TTTGATTAC CAAGCTATCA AAATGGTCAG ACACTGTGCT GCAATGCACA ATTGGAGCAT 660  
TTCACATGCG TTGCATGAAT TATTCCTTGG GTTAGGAAAC CTTTGAAATA CCTTGACTAA 720  
20 GGTAAAAAAA AAACTTGAC AAATTAATA ATATTAATAT TGATTTTGTA CGTACACGAC 780  
TTAACCAAAC TCTCAATGAT TTATTGATT CTAATATATA TATTAATAAC GTACGTCTAA 840  
25TTGGATCATT CATGATCTAC AGCCATCACA TCTCAGATGA TTTTCTTGCA ATGAATTGCC 900  
TAAGCTGGCG TTATTATCTT TTTTTCATAA TACAGTTTA AAAAAGGGTA CGTATTGGAG 960  
CTGGTGATGA CTTCTTAAGA AACAACAAAT TAACGCCATA GCTATTTGAT TTATATATCC 1020  
30 AAAAGGAGAA AATGTATAAG ATCGTTGCTT ACTTAATTG CAGGCTAGGT TAATTGACAT 1080  
CAAATAATTG AAGAGTACGT AGGGCCAATG TTGCTGAGAT CTAGCATCAA TAATAGGATT 1140  
35TGGCTTGTCG ATCGATCATC TTTATTTAAT TGAGAGGTAT GTATCCATAT GTTTTCTGAA 1200  
ATTAAAATAT TACCTAATAA TTGAGCTGAA ACTGTAGTGA ATTTAACCTT TTCTAAGTTC 1260  
TGCCCATATA TAACATACCA CATAGGTAGC TGATCGATCG ATCATATATA TGTACTTAGG 1320  
40 GTTCTGATCA GTATCAATAT CGATCACAAG TGCTGATAAT TAACCATGGC TCAATTCAAT 1380  
ATCCCAGTAA CCATGTCTTC AAGCTTAAGC ATAATTTTGG TCATTCTTGT ATCTTTGAGA 1440  
45ACTGCACTCT CAGAGCTATG CAACCCACAA GATAAGCAAG CCCTTCTCCA AATCAAGAAA 1500

	GACCTTGGCA ACCCAACCAC TCTCTCTTCA TGGCTTCCAA CCACCGACTG TTGTAACAGA	1560
	ACCTGGCTAG GTGTTTTATG CGACACCGAC ACCCAAACAT ATCGCGTCAA CAACCTCGAC	1620
	5CTCTCCGGCC ATAACCTCCC AAAACCCTAC CCTATCCCTT CCTCCCTCGC CAACCTCCCC	1680
	TACCTCAATT TTCTATACAT TGGCGGCATC AATAACCTCG TCGGTCCAAT CCCCCCGCC	1740
10	ATCGCTAAAC TCACCCAACT CCACTATCTC TATATCACTC ACACCAATGT CTCCGGCGCA	1800
	ATACCCGATT TCTTGTCA CA GATCAAAACC CTCGTCACCC TCGACTTCTC CTACAACGCC	1860
	CTCTCCGGCA CCCTCCCTCC CTCCATCTCT TCTCTCCCA ACCTCGGAGG AATCACATTC	1920
15	GACGGCAACC GAATCTCCGG CGCCATCCCC GACTCCTACG GCTCGTTTTT GAAGCTGTTT	1980
	ACGGCGATGA CCATCTCCCG CAACCGCCTC ACCGGGAAGA TTCCACCGAC GTTTGCGAAT	2040
20	CTGAACCTGG CGTTCGTTGA CTTGTCTCGG AACATGCTGG AGGGTGACGC GTCGGTGTTG	2100
	TTCGGGTCAG ATAAGAACAC GAAGAAGATA CATCTGGCGA AGAACTCTCT TGCTTTTGAT	2160
	TTGGGGAAG TGGGGTTGTC AAAGAACTTG AACGGGTTGG ATCTGAGGAA CAACCGTATC	2220
25	TATGGGACGC TACCTCAGG ACTAACGCAG CTAAAGTTT TGCAAAGTTT AAATGTGAGC	2280
	TTCAACAATC TGTGCGGTGA GATTCCTCAA GGTGGGAACT TGAAAAGGTT TGACGTTTCT	2340
30	TCTTATGCCA ACAACAAGTG CTTGTGTGGT TCTCCTCTTC CTTCTGCAC TTAACCATTT	2400
	CCAGATTCCG TAATTATGGA TGCATCATGT TTGCCTTTCT ATGAACATCA ATAATGATAC	2460
	AAGTGTA AAA ATAAAAATAA ATTTATGATA TATAATAAAC GTCTTGATC ATTATTTT	2520
35	TCCTAAAGTG AATTATAATA TTTGCTGATA AAAAAAGCT CTCTCTCATA GGTAAGTATA	2580
	TTTTTTAATA CATTGACTG AAATAACATA TTCTCTGTAT GTACGTCGTA CTTAGGATCC	2640
40	CCCGGGCTGC AGATCGTTCA AACATTGGC AATAAGTTT CTTAAGATTG AATCCTGTTG	2700
	CCGGTCTG C GATGATTATC ATATAATTTC TGTGAATTA CGTTAAGCAT GTAATAATTA	2760
	ACATGTAATG CATGACGTTA TTTATGAGAT GGGTTTTTAT GATTAGAGTC CCGCAATTAT	2820
45	ACATTTAATA CGCGATAGAA AACAAAATAT AGCGCGCAA CTAGGATAAA TTATCGCGCG	2880

CGGTGTCATC TATGTTACTA GATCTTCTAG AAAGCTT

2917

## (2) INFORMATION FOR SEQ ID NO:21:

## 5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 342 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## 10 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

15 (C) INDIVIDUAL ISOLATE: predicted amino acid coding sequence  
of SEQ ID NO:20

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

20

Met Ala Gln Phe Asn Ile Pro Val Thr Met Ser Ser Ser Leu Ser Ile  
1 5 10 15

25

Ile Leu Val Ile Leu Val Ser Leu Arg Thr Ala Leu Ser Glu Leu Cys  
20 25 30Asn Pro Gln Asp Lys Gln Ala Leu Leu Gln Ile Lys Lys Asp Leu Gly  
35 40 45

30

Asn Pro Thr Thr Leu Ser Ser Trp Leu Pro Thr Thr Asp Cys Cys Asn  
50 55 60Arg Thr Trp Leu Gly Val Leu Cys Asp Thr Asp Thr Gln Thr Tyr Arg  
65 70 75 80

35

Val Asn Asn Leu Asp Leu Ser Gly His Asn Leu Pro Lys Pro Tyr Pro  
85 90 95

40

Ile Pro Ser Ser Leu Ala Asn Leu Pro Tyr Leu Asn Phe Leu Tyr Ile  
100 105 110Gly Gly Ile Asn Asn Leu Val Gly Pro Ile Pro Pro Ala Ile Ala Lys  
115 120 125

45

Leu Thr Gln Leu His Tyr Leu Tyr Ile Thr His Thr Asn Val Ser Gly



48

	130		135		140
	Ala Ile Pro Asp Phe Leu Ser Gln Ile Lys Thr Leu Val Thr Leu Asp				
	145		150		155 160
5	Phe Ser Tyr Asn Ala Leu Ser Gly Thr Leu Pro Pro Ser Ile Ser Ser				
		165		170	175
	Leu Pro Asn Leu Gly Gly Ile Thr Phe Asp Gly Asn Arg Ile Ser Gly				
10		180		185	190
	Ala Ile Pro Asp Ser Tyr Gly Ser Phe Ser Lys Leu Phe Thr Ala Met				
		195		200	205
15	Thr Ile Ser Arg Asn Arg Leu Thr Gly Lys Ile Pro Pro Thr Phe Ala				
		210		215	220
	Asn Leu Asn Leu Ala Phe Val Asp Leu Ser Arg Asn Met Leu Glu Gly				
20		225		230	235 240
	Asp Ala Ser Val Leu Phe Gly Ser Asp Lys Asn Thr Lys Lys Ile His				
		245		250	255
	Leu Ala Lys Asn Ser Leu Ala Phe Asp Leu Gly Lys Val Gly Leu Ser				
25		260		265	270
	Lys Asn Leu Asn Gly Leu Asp Leu Arg Asn Asn Arg Ile Tyr Gly Thr				
		275		280	285
30	Leu Pro Gln Gly Leu Thr Gln Leu Lys Phe Leu Gln Ser Leu Asn Val				
		290		295	300
	Ser Phe Asn Asn Leu Cys Gly Glu Ile Pro Gln Gly Gly Asn Leu Lys				
35		305		310	315 320
	Arg Phe Asp Val Ser Ser Tyr Ala Asn Asn Lys Cys Leu Cys Gly Ser				
		325		330	335
	Pro Leu Pro Ser Cys Thr				
40		340			

(2) INFORMATION FOR SEQ ID NO:22:

(1) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 1356 base pairs

(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Exemplary drul promoter sequence

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGCATATCA ACAACTACGA ATAAAGAGAT CAGCCTTTCC GTATCTGGTG GATGTTTGAG	60
TCGGTGATGA CCATCTAATT AAAGAAAGAA GAAAAATTAT ACATATTGTG GACCTCCCCA	120
20 TATATAATTC TTATCATCTT TGTTACTGCC ATTATGATTA TAAATGATA TTAAAGGGAT	180
GGTGTACCGT GTACTAATCA AATATCTACC TGATCTTATT GATTTGAAAG ATCATAAAAA	240
25 GAAATTAAAA TTGTTCAAAA TAAACCCCTA GAATTATATA TAGTTCATTA AGTTCAAATT	300
AATTCGTTTG AAACGTGTTA AGCAACCCTA CAACGTACTA AGCACCCTAG CTCCCTTTGC	360
CTCTCGGCGG TAAGAGGAGA TATCCTCAGT CGAATTATGA GCCGATCGAG GAAAGCTCGA	420
30 TCAGTTGGAA AATCTTTCTT TCTTATGGCC AAGTTGTTTC AAACAATATA TTGAATTATT	480
GACTCTTAGC AACTTAAGTT TCAAACCGTG ACGAACCAAT AAAATTTGAC AAATTAATCA	540
35 CTTTAAGTGC CTAGTGGATC AGCGTCTAGG TTGGGAACCC CTCTACCTGC GTTGATTCA	600
CCAAGCTATC AAAATGGTCA GACACTGTGC TGCAATGCAC AATTGGAGCA TTTCACATGC	660
GTTGCATGAA TTATTCCTTG GGTTAGGAAA CCTTGAAAT ACCTTGACTA AGGTAAAAAA	720
40 AAAAACTTGA CAAATTAATA AATATTAATA TTGATTTTGT ACGTACACGA CTTAACCAAA	780
CTCTCAATGA TTTATTGATT TCTAATATAT ATATTAATAA CGTACGTCTA ATTGGATCAT	840
45 TCATGATCTA CAGCCATCAC ATCTCAGATG ATTTTCTTGC AATGAATTGC CTAAGCTGGC	900

GTTATTATCT TTTTTCATA ATACAGTTTT AAAAAAGGGT ACGTATTGGA GCTGGTGATG 960  
ACTTCTTAAG AAACAACAAA TTAACGCCAT AGCTATTGGA TTTATATATC CAAAAGGAGA 1020  
5AAATGTATAA GATCGTTGCT TACTTAATTT GCAGGCTAGG TTAATTGACA TCAAATAATT 1080  
GAAGAGTACG TAGGGCCAAT GTTGCTGAGA TCTAGCATCA ATAATAGGAT TTGGCTTGTC 1140  
GATCGATCAT CTTTATTTAA TTGAGAGGTA TGTATCCATA TGTTTTCTGA AATTAAAATA 1200  
10 TTACCTAATA ATTGAGCTGA AACTGTAGTG AATTAACTT TTTCTAAGTT CTGCCCATAT 1260  
ATAACATACC ACATAGGTAG CTGATCGATC GATCATATAT ATGTACTTAG GGTCTGATC 1320  
15AGTATCAATA TCGATCACAA GTGCTGATAA TTAAAC 1356

## IT IS CLAIMED:

1. A chimeric gene, comprising  
(i) a DNA sequence encoding a product of interest, and (ii) a *dru1* promoter, where said  
5 DNA sequence is heterologous to said promoter and said DNA sequence is operably linked to said  
promoter to enable expression of said product.
2. A chimeric gene of claim 1, wherein said DNA sequence encodes a product selected from  
the group consisting of S-adenosylmethionine hydrolase, aminocyclopropane-1-carboxylic acid  
10 (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC  
oxidase cosuppression molecule, and ACC synthase cosuppression molecule.
3. A chimeric gene of claim 1, wherein said DNA sequence is a pathogenesis related gene.
- 15 4. A chimeric gene of claim 3, wherein said DNA sequence is selected from the group  
consisting of polygalacturonase inhibiting protein (PGIP), glucanase and chitinase.
5. A chimeric gene of claim 1, wherein said DNA sequence encodes a product selected from  
the group consisting of thaumatin, sucrose phosphate synthase and lycopene cyclase.  
20
6. A chimeric gene of claim 1, wherein the promoter is obtained from a gene homologous to  
a raspberry *dru1* gene.
7. A chimeric gene of claim 6, wherein the promoter is from a raspberry *dru1* gene.  
25
8. A chimeric gene of claim 7, wherein the promoter is derived from the sequence presented  
as SEQ ID NO:22.
9. A plant transformation vector containing the chimeric gene of any of claims 1-8.  
30
10. A kit for use in plant transformation, comprising the vector of claim 9.
11. A plant cell containing the chimeric gene of any of claims 1-8.

12. A transgenic fruit-bearing plant, comprising  
the chimeric gene of any of claims 1-8.
13. A fruit produced by the plant of claim 12.
- 5 14. A method for modifying ripening fruit of a fruit bearing plant, comprising,  
growing the plant of claim 12, to produce a transgenic plant bearing fruit, wherein (i) the  
chimeric gene encodes a product capable of reducing ethylene biosynthesis when expressed in plant  
cells, and (ii) fruit produced by said plant has a modified ripening phenotype.
- 10 15. A method for producing a transgenic fruit-bearing plant, comprising  
introducing into progenitor cells of the plant a chimeric gene of any of claims 1-8, and  
growing the transformed progenitor cells to produce a transgenic plant bearing fruit.
- 15 16. A method of claim 15, where said introducing includes transforming progenitor cells of the  
plant with a selectable vector containing said chimeric gene.
17. A method of claim 15, wherein the promoter is isolated by the steps of:  
(i) selecting a probe DNA molecule containing a sequence homologous to a region of  
20 raspberry *drul* gene DNA,  
(ii) contacting the probe with a plurality of target DNA molecules derived from the genome  
of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe  
molecule and a target molecule homologous to the probe molecule,  
(iii) identifying a target molecule having a DNA sequence homologous to the raspberry *drul*  
25 gene, and  
(iv) isolating promoter sequences associated with the target molecule.
18. A method of isolating a *drul* promoter, comprising  
(i) selecting a probe DNA molecule containing a sequence homologous to a region of  
30 raspberry *drul* gene DNA,  
(ii) contacting the probe with a plurality of target DNA molecules derived from the genome  
of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe  
molecule and a target molecule homologous to the probe molecule,

(iii) identifying a target molecule having a DNA sequence homologous to the raspberry *dru1* gene, and

(iv) isolating promoter sequences associated with the target molecule.

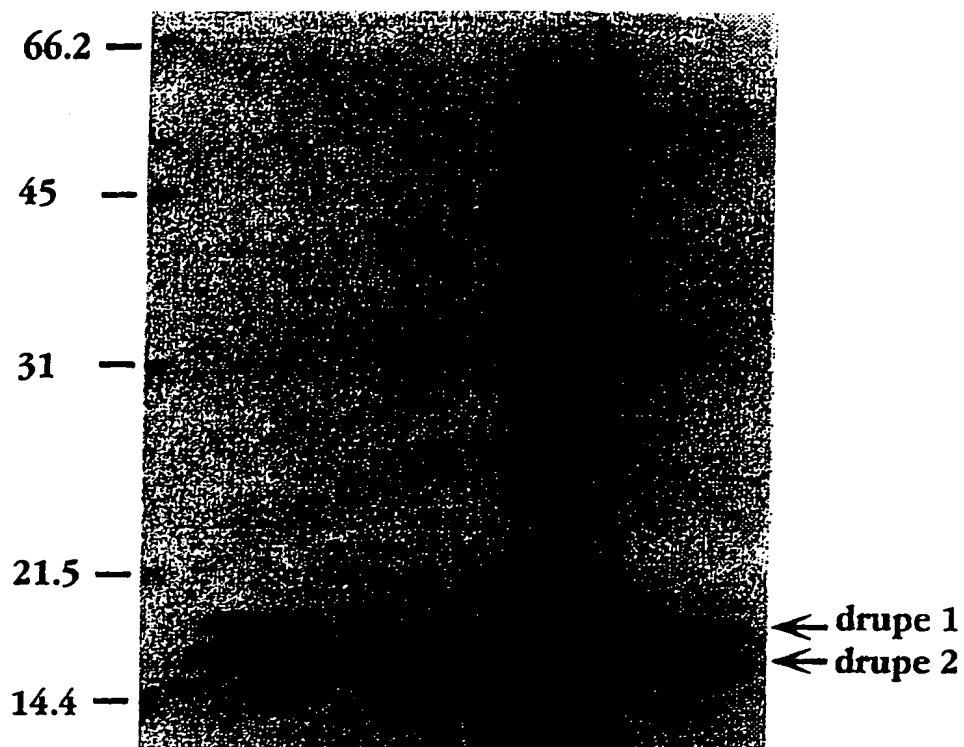
5 19. A method of claim 18, where said probe DNA molecule has the sequence presented as SEQ ID NO:22.

20. A method of claim 18, where said fruit-bearing plant is selected from the group consisting of grapes, strawberries, blackberries, plums, cherries, peaches, blueberries and cranberries.

10

21. An isolated DNA molecule comprising a *dru1* promoter.

22. An isolated DNA molecule comprising a promoter from a raspberry *dru1* gene.



**Fig. 1**

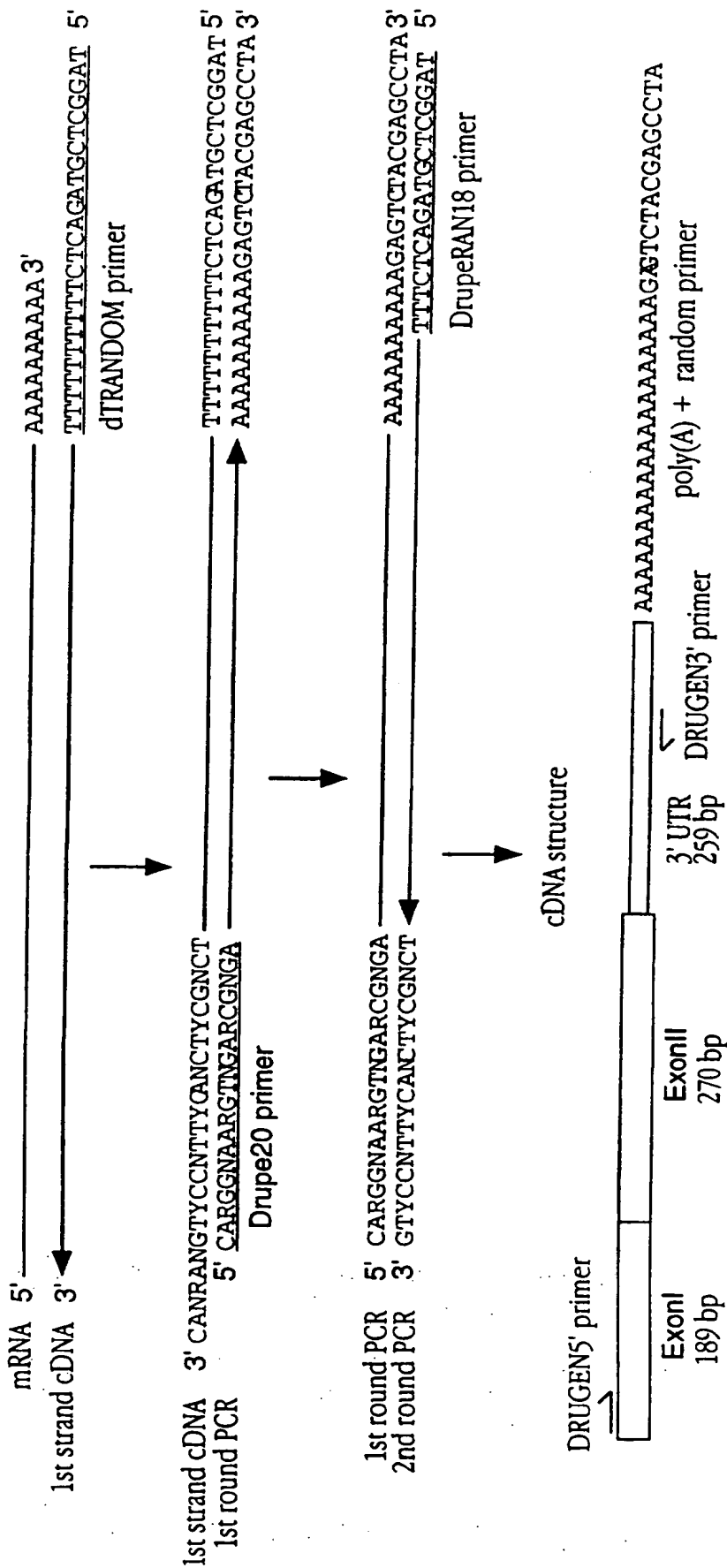
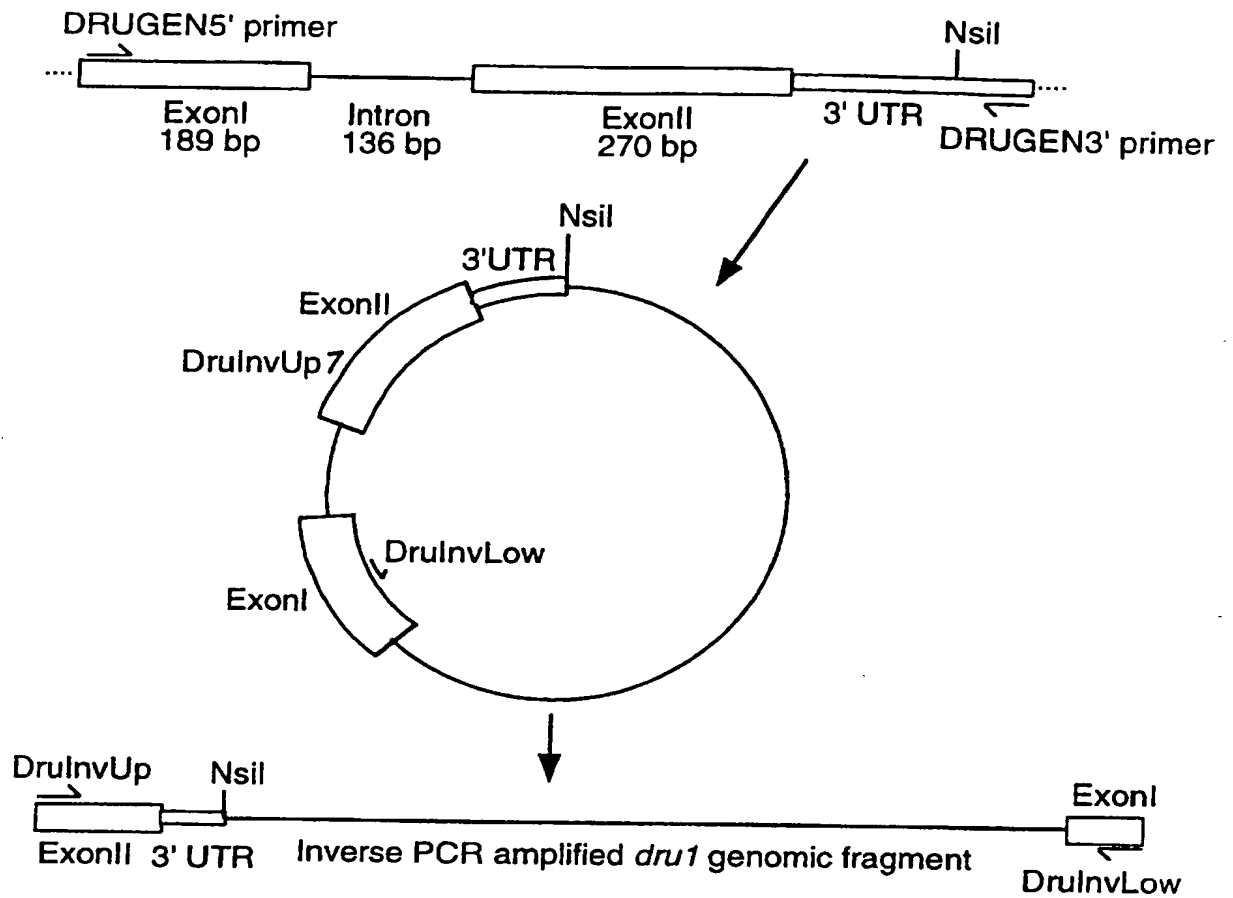


Fig. 2A



3/10

**Fig. 2B**

4/10

&gt;Nsi\_I

```

      10      20      30      40      50      60      70      80
ATGCATATCA ACAACTACGA ATAAAGAGAT CAGCCTTTCC GTATCTGGTG GATGTTTGAG TCGGTGATGA CCATCTAATT
      90     100     110     120     130     140     150     160
AAAGAAAGAA GAAAAATTAT ACATATTGTG GACCTCCCCA TATATAATTC TTATCATCTT TGTACTGCC ATTATGATT
     170     180     190     200     210     220     230     240
TAAATGATA TTAAAGGGAT GGTGTACCGT GTACTAATCA AATATCTACC TGATCTTATT GATTTGAAAG ATCATAAAAA
     250     260     270     280     290     300     310     320
GAAATTAAAA TTGTTCAAAA TAAACCCCTA GAATTATATA TAGTTCATTA AGTTCAAATT AATTCGTTTG AAACGTGTTA
     330     340     350     360     370     380     390     400
AGCAACCCCTA CAACGTACTA AGCACCCCTAG CTCCTTTGCG CTCTCGGGG TAAGAGGAGA TATCCTCAGT CGAATTATGA
     410     420     430     440     450     460     470     480
GCCGATCGAG GAAAGCTCGA TCAGTTGGAA AATCTTTCTT TCTTATGGCC AAGTTGTTTC AAACAATATA TTGAATTATT
     490     500     510     520     530     540     550     560
GACTCTTAGC AACTTAAGTT TCAACCGTG ACGAACCAAT AAAATTTGAC AAATTAATCA CTTTAAGTGC CTAGTGGATC
     570     580     590     600     610     620     630     640
AGCGTCTAGG TTGGGAACCC CTCTACCTGC GTTTGATTCA CCAAGCTATC AAAATGGTCA GACACTGTGC TCGAATGCAC
     650     660     670     680     690     700     710     720
AATTGGAGCA TTTCATATGC GTTCATGAA TTATTCCTTG GGTAGGAAA CCTTTGAAAT ACCTTGACTA AGGTAAAAAA
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AAAACTTGA CAAATTAATA AATATTAATA TTGAATTTGT ACGTACACGA CTTAACCAAA CTCTCAATGA TTTATTGATT
     810     820     830     840     850     860     870     880
TCTAATATAT ATATTAATAA NGTANGTCTA ATTGGATCAT TCATGATCTA CAGCCATCAC ATCTCAGATG ATTTTCTTGC
     890     900     910     920     930     940     950     960
AATGAATTGC CTAAGCTGCG GTTATTATCT TTTTTCATA ATACAGTTT AAAAAGGGT ACGTATTGGA GCTGGTGATG
     970     980     990    1000    1010    1020    1030    1040
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     1130    1140    1150    1160    1170    1180    1190    1200
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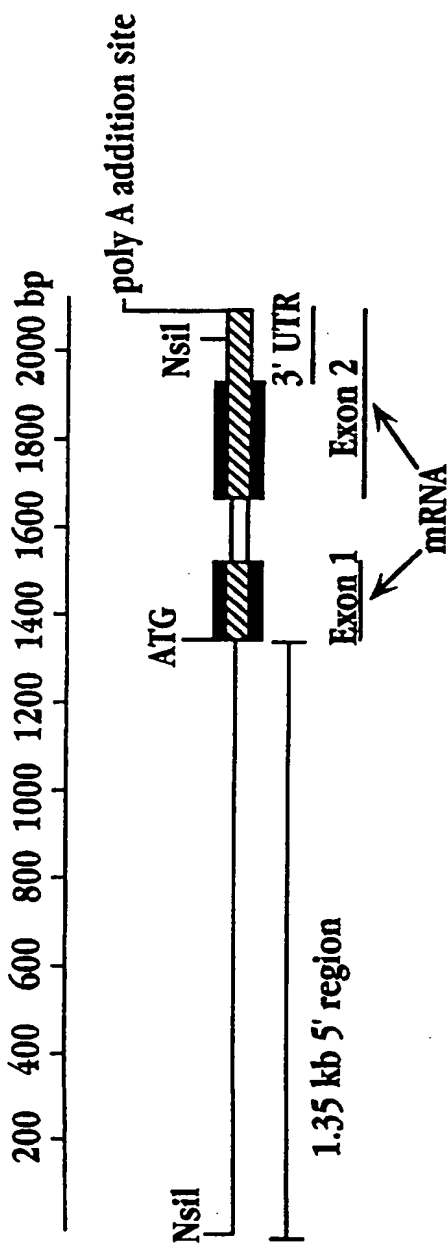
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Fig. 3A

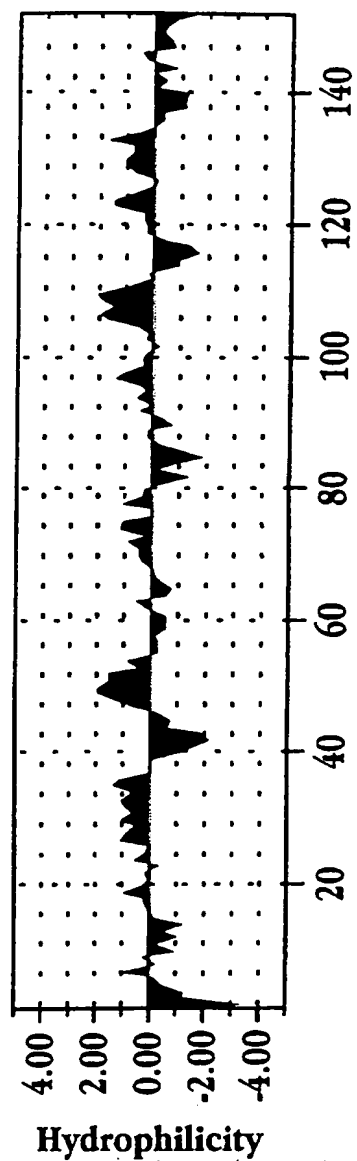
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**Fig. 3B**

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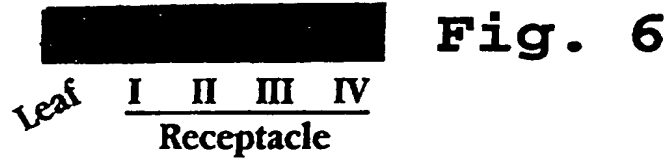


**Fig. 4**

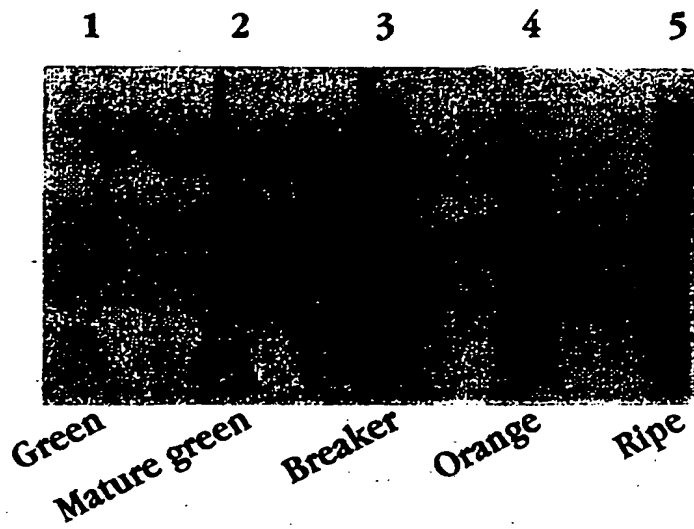
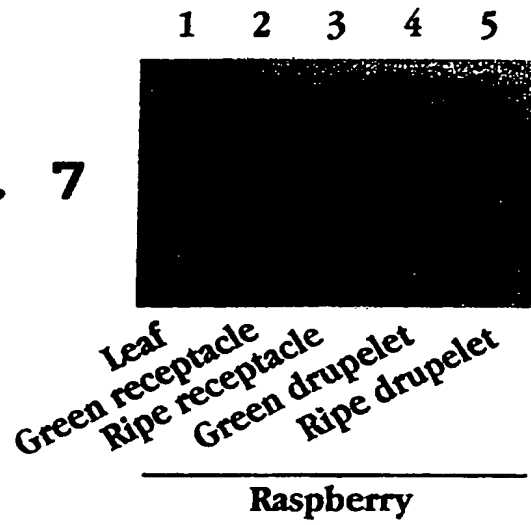


**Fig. 5**

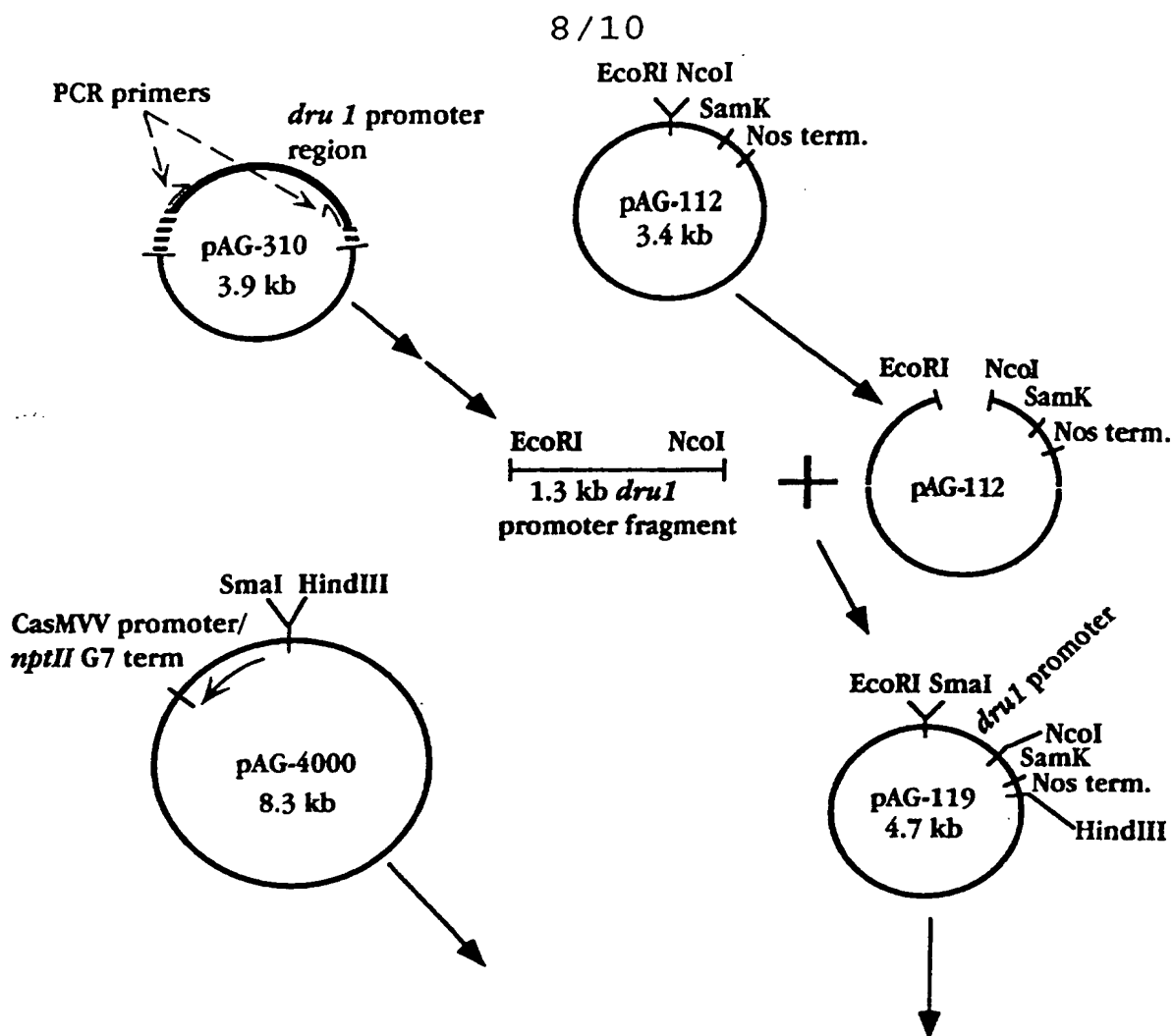
7/10

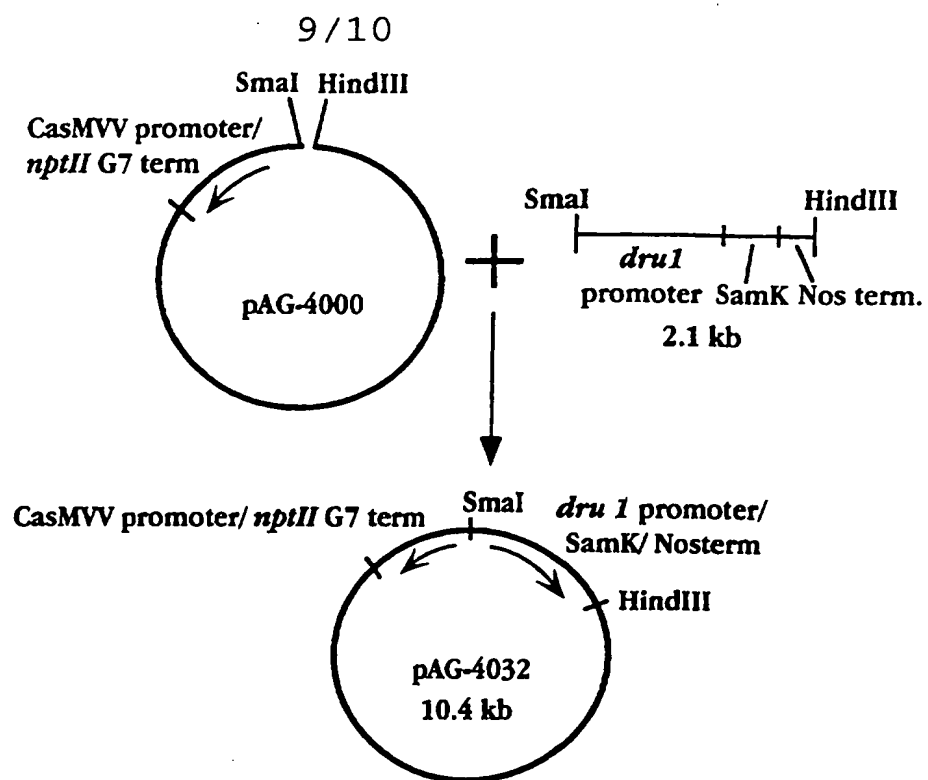


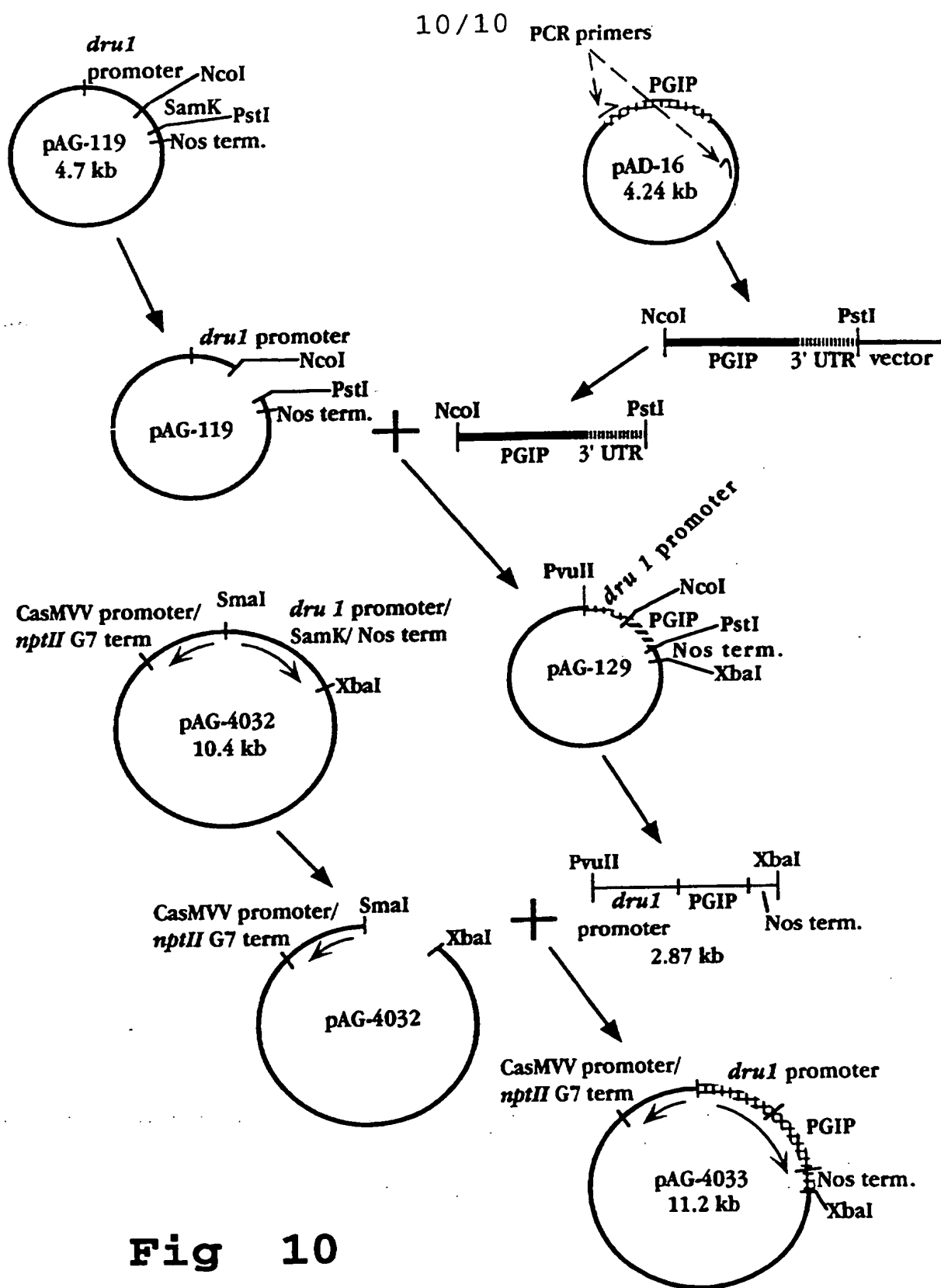
**Fig. 7**



**Fig. 8**

**Fig. 9A**

**Fig. 9B**

**Fig 10**



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT, JS 97/01443

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 35388 A (EPITOPE INC) 28 December 1995 see page 7, line 1 - line 4 see page 12, line 29 - line 32 see page 14, line 33 - page 15, line 12 see page 21, line 1 - page 22, line 5 ---	1-22
A	WO 95 35387 A (EPITOPE INC) 28 December 1995 see page 17, line 9 - line 12 see page 22, line 25 - line 32 see page 52, line 8 - page 54, line 5 --- -/--	1-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

21 May 1997

Date of mailing of the international search report

23.05.97

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/JS 97/01443

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ACTA HORTICULTURAE, (1993) NO. 352, PP. 601-606. 17 REF. MEETING INFO.: SIXTH INTERNATIONAL SYMPOSIUM ON RUBUS AND RIBES HELD AT SKIERNIEWICE, POLAND, 3-10 JULY, 1993., XP000671960</p> <p>WILLIAMSON, B. ET AL: "A polygalacturonase inhibitor from immature raspberry fruits: a possible new approach to grey mould control" see the whole document</p> <p>---</p>	4
A	<p>WO 93 11241 A (CONSIGLIO NAZIONALE RICERCHE ;UNIV GEORGIA (US)) 10 June 1993 see the whole document</p> <p>---</p>	4
A	<p>PLANT MOLECULAR BIOLOGY 26 (3). 1994. 781-790. , XP002031050</p> <p>GOOD X ET AL: "Reduced ethylene synthesis by transgenic tomatoes expressing S-adenosylmethionine hydrolase." see the whole document</p> <p>---</p>	2
A	<p>PLANT MOLECULAR BIOLOGY, vol. 28, 1995, pages 1011-1025, XP002031051</p> <p>POZUETA-ROMERO, J., ET AL.: "Characterization of a family of genes encoding a fruit-specific wound-stimulated protein of bell pepper (Capsicum annuum): identification of a new family of transposable elements" see the whole document</p> <p>---</p>	1
A	<p>EMBL SEQUENCE DATABASE, REL. 46 06-DEC-1995, ACCESSION NO. X91960, XP002031030</p> <p>GRELLET, F., ET AL.: "A.thaliana mRNA for major latex protein type 1" see sequence</p> <p>-----</p>	1

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International Application No

PCT/US 97/01443

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